Investigation of Tumor Frame Shift Antigens for Prophylactic Cancer Vaccine, Cancer Detection and Tumorigenicity

by

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ABSTRACT

Cancer is one of the most serious global diseases. We have focused on cancer immunoprevention. My thesis projects include developing a prophylactic primary and metastatic cancer vaccines, early cancer detection and investigation of genes involved in tumor development. These studies were focused on frame-shift (FS) antigens. The FS antigens are generated by genomic mutations or abnormal RNA processing, which cause a portion of a normal protein to be translated out of frame.

The concept of the prophylactic cancer vaccine is to develop a general cancer vaccine that could prevent healthy people from developing different types of cancer. We have discovered a set of cancer specific FS antigens. One of the FS candidates, structural maintenance of chromosomes protein 1A (SMC1A) FS, could start to accumulate at early stages of tumor and be specifically exposed to the immune system by tumor cells. Prophylactic immunization with SMC1A-FS could significantly inhibit primary tumor development in different murine tumor models and also has the potential to inhibit tumor metastasis.

The SMC1A-FS transcript was detected in the plasma of the 4T1/BALB/c mouse tumor model. The tumor size was correlated with the transcript ratio of the SMC1A-FS verses the WT in plasma, which could be measured by regular RT-PCR. This unique cancer biomarker has a practical potential for a large population cancer screen, as well as clinical tumor monitoring.

With a set of mimotope peptides, antibodies against SMC1A-FS peptide were detected in different cancer patients, including breast cancer, pancreas cancer and lung cancer with a 53.8%, 56.5% and 12.5% positive rate respectively. This suggested that the FS antibody could be a biomarker for early cancer detection.

The characterization of SMC1A suggested that: First, the deficiency of the SMC1A is common in different tumors and able to promote tumor initiation and development; second, the FS truncated protein may have nucleolus function in normal cells. Mis-control of this protein may promote tumor development.

In summary, we developed a systematic general cancer prevention strategy through the variety immunological and molecular methods. The results gathered suggest the SMC1A-FS may be useful for the detection and prevention of cancer.

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CHAPTER 1

INTRODUCTION

1.1 Cancer Statistics

Cancer is one of the most serious global diseases threatening human lives. It is one of the leading causes of death in both developing and developed countries. In developed countries, for example in the US, from 1999 to 2008, the cancer death rate is decreasing more than 1% per year. However the over all tumor incident rate is increasing in both male and female. In developing countries, although cancer incident rate is about half of the developed countries, the overall cancer death rate is at a similar level [1, 2]. There were over 12 million new cases of cancer diagnosis and 7.6 million deaths from cancer in the world in 2008. This is more than doubled in the past 30 years. What is worse, the global cancer burden will reach 27 million annual incidents and 17 million deaths by 2030[3].

This rapidly increasing cancer burden hugely impacts public health and society. Cancer causes the highest economic loss of any disease in the world. The global economic impact of premature death and disability from cancer was $895 billion in 2008, which is 1.5% of the world's gross domestic product. This was not including the direct cost of the cancer treatment. In the US, the direct medical cost of cancer was estimated to be $103.8 billion in 2007 [4]. The global economic loss by cancer is projected to increase along with the increasing of the cancer incident and death.

This huge global cancer burden is mainly caused by the increase in the life expectancy of individuals and populations of the world. This is also caused by an increase in cancer risk behaviors, such as tobacco use, alcohol use, unhealthy diet and physical inactivity. For example, tobacco use causes 22% of cancer deaths and 71% lung cancer death in the world[3]. The regulation and education of the tobacco use steadily decreased the smoking rate in the US. The reduction of the smoking rate contributed to the decline of both incidence and mortality of lung cancer in men in the US starting in the 1990s. It has been estimated that about 30% of cancer burden can be reduced by outreach programs focused on education of current cancer control knowledge and enforcement to modify or avoid these key cancer risk factors[3]. Therefore, more public education and regulation leads to great cancer incidence and survival control.

1.2 Cancer treatment

Cancer control through medical care could be simply classified into three areas: cancer treatment, cancer prevention and cancer detection.

The traditional cancer treatment includes three main methods: surgery, radiotherapy and chemotherapy. More recently, cancer immunotherapy has started to show promise and become an important component for cancer treatment.

Surgery was and remains the first available method for cancer treatment by removing the tumor from the patients. This started at the beginning of the 19th century and fast developed after the introduction of anesthesia and antisepsis in the middle of the 19th century. Although few cancer patients could be cured by surgical treatment alone, surgery was the only option for most cancer patients until the development of radiotherapy by the middle of the 20th century.

Cancer radiotherapy started at the end of the 19th century after the discovery of x-rays by Roentgen. Radiotherapy was further developed during the 20th century, along with the development of new technologies, such as radiators and computer science. The cancer cure rate with surgery, radiotherapy or a combination was increased by about 30% in the 1950s[5].

At the same time, the chemotherapy started to develop by screening chemicals to target cancer cells. By the middle of the 1960s, it was proven that combination chemotherapy could cure childhood leukemia and advanced Hodgkin's lymphoma [6, 7]. Since then, more drugs were discovered to treat major cancers, such as breast, colorectal, lung cancers and melanoma.

The first clinical trial of cancer immunotherapy started in 1891 by William Coley, who first developed 'Coley's toxins' to treat developed erysipelas with live or inactive bacteria [8]. However, the efficacy of this type of treatment and other immunotherapy trials, including therapeutic cancer vaccines, was poor. Cancer immunotherapy was not accepted as the general clinical treatment until recent decades with the accumulation of the knowledge of the immune system and tumor

cells. For example, nine monoclonal antibodies have been approved for the treatment of solid tumors and lymphoma caner. These monoclonal antibodies target cancer associated proteins, such as epidermal growth factor receptors and vascular endothelial growth factors, and induce tumor cell apoptosis, antibody-dependent cellular cytotoxicity and phagocytosis and may also elicit adaptive immune response against tumor cells [9]. More recently, the first therapeutic cancer vaccine was also approved by the US FDA to treat asymptomatic metastatic castrate-resistant prostate cancer. There are currently many cancer immunotherapies in clinical trials targeting a variety of pathways, such as pathways regulating immune checkpoints and targeting different tumor associated antigens. Cancer immunotherapy is becoming another effective method of cancer treatment.

With the development of new cancer treatments, it is clear that the single cancer treatments could not effectively cure cancer. With a combination of different cancer treatments and improved diagnostic methods, the mortality rate of both breast and colorectal cancer was successfully decreased during past decades, especially in developed countries, which have advanced cancer treatment resources. However, the complex characteristic of cancer, such as metastasis and immune suppression, limit the efficacy of the current cancer treatments, especially for cancer at late stages. Additionally, the costs of current efficient cancer treatments are expensive. This also limits the contributions of the advanced cancer treatments to the global cancer control efforts, especially in developing countries.

1.3 Cancer Prevention

Although the developments of cancer treatment have successfully controlled the mortality of cancer in developed countries, the overall cancer incidence is still increasing since 1970s, even in developed countries. Despite the advanced achievements in cancer treatments, cancer prevention is still considered to be the most cost-effect long term goal for global cancer control. However, compared to the impressive developments in cancer treatment, the development of cancer prevention through medical care has made little advancements. There were only two notable successes in this area so far: chemoprevention and cancer related virus vaccines.

It was clearly shown that the chemical drugs used for cancer treatment could also effectively prevent specific cancers, such as finasteride for prostate cancer prevention and antiestrogens for breast cancer prevention. Recently, colorectal cancer prevention by aspirin also has been demonstrated[10]. However, because of the potential for toxicity and serious side effects, current effective chemoprevention drugs cannot be widely used for cancer prevention in the healthy population.

The development of vaccines against liver cancer related hepatitis B virus (HBV) and cervical cancer related human papilloma virus (HPV) are another two successful achievements of cancer prevention [11, 12]. These vaccines, so called prophylactic cancer vaccines, are actually traditional infectious disease vaccines. HBV vaccine has been proven to reduce 49% liver cancer incidence among

immunized children and 42% liver cancer risk of immunized adults. It has been estimated that about 17.8% cancer incidents are related to pathogen infections [13]. Therefore, the development of vaccines against cancer related virus is an important strategy for cancer prevention and needs to be further explored.

1.4 Dynamic Interaction between immune system and tumor

1.4.1 Cancer Immune Surveillance

In 1909, Ehrlich proposed the original hypothesis that the immune system can also recognize and destroy nascent transformed cells in our body and prevent them from manifesting clinically[14]. Fifty years later, Burnet and Thomas revisited this idea and proposed a formal hypothesis of immune surveillance: thymus dependent cells constantly protect host from nascent transformed cells through effective immune response to tumor specific antigens [15, 16]. Based on this hypothesis, the tumor incident should increase with the deficiency of the immune system. However, this hypothesis was not supported by work which was performed by Stutman and others in the 1970s. They demonstrated that compared to normal mice, the tumor incidence did not increase in athymic-nude mice, mice with a genetic deficit causing failure of normal development of the thymus and therefore, are lacking mature T cells[17]. Now we know that the athymic-nude mice are not the appropriate model to test the hypothesis. The main hindrance of the athymice-nude mice is that they are not completely immunodeficient. Immune mechanisms, other than the mature T cells, involving cancer immune surveillance

are marginally distributed in these nude mice, such as innate immune response, and a few T cells could still mature even without a proper thymus environment. Therefore, the immune surveillance still could efficiently monitor tumor development and contribute to tumor prevention during the time periods of these experiments. On the other hand, these experiments suggest that efficacy of the immune surveillance in tumor prevention. Even the impaired immune system could efficiently prevent the tumor development for a relatively long time. However, this hypothesis was seriously challenged at that time.

After the 1990s, the immune surveillance of cancer was validated with new technologies through a variety of well documented experiments using different systems, such as dysfunction of specific immune responses by modifying specific genes in mice, inhibition of different immunologic components by different monoclonal antibodies and stimulation of specific immune responses by different activators. For example, the treatment with monoclonal antibody against IFN-γ could block the LPS induced tumor rejection in both transplanted and chemical induced mouse tumor models[18]. The IFN-$\gamma^{-/-}$ C57BL6 mice and BALB/c mice displayed increased incidence of disseminated lymphomas and spontaneous lung adenocarcinomas respectively [19, 20]. IFN-γ is a critical cytokine for both innate and adaptive immune responses. Finally, the cancer immune surveillance was directly proven in the appropriate immunodeficient mouse models, such as BALB/c SCID mice, RAG-$1^{-/-}$ and RAG-$2^{-/-}$ mice [21-23]. The B cell, T cell and NKT cells in these mice were

dysfunctional by genetic modification. The chemical induced tumor incidents in these mice were significantly increased compared to wild type mice. The theory of cancer immune surveillance is also substantiated by the fact that both the innate and adaptive immune systems are involved in the immune surveillance of cancer.

1.4.2 Cancer Immune Evolving

The immune surveillance hypothesis has now been supported for decades and suggests that the immune system can control tumor development. However, this still could not explain the fact that most cancers initiate in healthy people with proper immune systems that can still efficiently fight infections. The accumulated evidence suggests that the one dimensional description of the immune system inhibiting tumor development by the theory of immune surveillance is not sufficient. The refined theory is described as cancer immunoediting and regulation to reflect the dynamic interaction between the immune system and tumor: immune surveillance inhibits tumor development from tumor initiation, while the tumor develops under the immune surveillance and evolves different mechanisms to avoid and resistant the immune attack or suppress the immune surveillance through multiple pathways, allowing the tumors to develop [24, 25].

Early studies had revealed that tumor cells from immunodeficient mice are more immunogenic than tumor cells from immunocompetent mice. For example, the chemical induced tumor cells from nude mice and SCID mice were more frequently rejected when transplanted into wild type mice, compared the chemical induced tumor cell from the wild type mice [26, 27]. This suggests that the

immune surveillance has the selective pressure contributing to tumor development and has forced tumor cells to evolve characteristics to escape the immune surveillance.

1.4.2.1 Cancer Immune Escape

The research on the molecular bases of tumor self-alteration revealed two major mechanisms: compromising of the antigen presentation pathway and resistance to apoptosis. Defects in MHC I antigen presentation are frequently found in mouse tumor models, as well as in 40%-90% of human tumor types [28, 29]. The loss of MHC I expression is the most frequent mechanism, which could be derived by either immune pressure or the oncogenic process. Garcia-Lora showed that an H2 class I negative fibrosarcoma cell line could generate H-2 negative lung metastasis in normal BALB/c mice, and generate H-2 class I positive lung metastasis in nude/nude mice [30-32]. This result reveals the selection of the immune surveillance. The loss of MHC I expression could be directly induced by tumor development, such as mutations or loss of heterozygosity (LOH) of MCH class genes with the genomic instability [33]and expression of oncogenes. It has been showed that over expression of HER2 could significantly reduce the expression of MHC class I in HER2 low expression human melanoma and breast carcinoma cell lines [34].

Apoptosis resistance was found in a variety of tumors with over-expression of anti-apoptosis proteins, such as FLICE-inhibitory proteins (FLIPs) and inhibitor of apoptosis proteins (IAPs)[35, 36]. For example, over-

expression of FLIP was detected in human melanomas and mouse tumor models [37-39]. FLIP has been shown to interfere with the death receptor induced apoptosis. The tumors with high expression of FLIP have been shown to be resistant to the T cell mediated immunity in vivo. On the other hand, the tumor apoptosis resistance could also be produced by deficiency of proapoptotic pathways. Down regulation and mutation of the death receptor, such as FasR, TRAIL-R1/2, were widely detected in different tumors, such as hepatocellular carcinomas, melanomas and lung cancers [40-43].

1.4.2.2 Cancer Immune Suppression

Besides developing mechanisms to escape the attacking immune system, tumor cells also develop a variety of mechanisms to suppress the immune surveillance both locally and systemically. A list of tumor derived immune suppression factors has been identified. These factors could either directly inhibit the infiltrated immune cells or recruit tumor associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and Regulatory T cells (Tregs) to the tumor microenvironment. All of such mechanisms consequently induce the anergy or apoptosis of activated anti-tumor immune cells and produce systemic tumor specific tolerance.

It has been found that the soluble Fas ligands (FasL) [44]and MHC class I polypeptide-related sequence A (MICA) [45]were increased in various tumors, such as lung, colon and breast tumors. The expression of soluble FasL in the tumor microenvironment could directly induce the filtrated T cell apoptosis

through the Fas receptor on the T cell surface. NKG2D is a receptor on NK cells and CD8 T cells. The soluble MICA could impair the NKG2D expression on the NK cell and T cell, and subsequently decrease the responsiveness of the tumor specific effector T cells [46]. The soluble MICA could not only suppress local T cells, but also impair the systemic T cell activity. The down regulation of the NKG2D on both tumor infiltrated T cells and peripheral blood T cells were observed in a variety of types of MICA positive cancer, including melanoma, breast, lung, ovarian and colon carcinomas [47, 48].

There are other tumor derived factors that could inhibit the infiltrated immune cells. The tumor-derived TGF-β could directly inhibit cytotoxic T cells through repressing the transcription of the cytotoxic genes, such as perforin, granzyme A and B, Fas ligand and IFN-γ [49]. The over-expression of serine protease inhibitor PI-9/SPI-6 in a variety of tumors could also inhibit the perfoin/granzyme B pathway of the cytotoxic T cells [50, 51]. Recently, it was found that the expression of B7-H1 on tumor cell surface could promote T cell apoptosis through programmed death-1 signal pathway[52].

The cellular immune suppression that is induced by tumors is involved in three types of cells: TAMs, MDSCs and Tregs. They are recruited or induced by different tumor derived factors and amplify these factors to further enhance the immune suppression microenvironment. They also could produce their unique factors directly to inhibit the immune cells.

TAMs could be recruited and accumulated in tumor microenvironment by tumor derived chemokines and cytokines, such as CC chemokines, soluble vascular endothelial growth factor (VEGF) and M-CFS that was released by tumor cell [53]. The TAMs are M2 polarized macrophages. In the tumor microenvironment, TAMs produce and excrete different molecular proteins, such as cytokines, chemokines, matrix proteins and proteases. Some of these could promote tumor development, such as by directly stimulating tumor growth, enhancing neoangiogenesis, changing tumor matix architecture and promoting tumor metastasis[54]. With respect to immune suppression, the TAMs derived IL-10, TGF-β and some of CC chemokines could inhibit the cytotoxic T cells and induce the Tregs by attracting naïve T cells to the tumor microenvironment [55, 56].

MDSCs recruited by tumor cells are highly heterogeneous, including immature granulocytes, macrophages and dendritic cells [57]. The heterogenous population of the MDSCs allows them to suppress the immune response with a variety of mechanisms. They could produce a similar set of the cytokines and chomkines as tumor cells or TAMs, such as IL-10, TGF-β, VEGF and CXCL5 [58], which could either directly suppress immune cells or recruit more immune suppression cells. Monocytic and granulocytic MDCSs could produce their unique extracellular molecules, such as nitic oxide and reactive oxygen species respectively [59, 60], and directly inhibit T cells. MDSCs also deprive the environment of arginine and cysteine, which are two essential amino acids for T

cell activation [61, 62]. MDSCs also could over express B7-1 on their surface and directly bind to the CTLA-4 on the activated T cells to induce a signal pathway that inactivates T cells [63].

Tregs are another important immune suppression cells for tumors to combat immune surveillance. The complex immune suppression factors in the tumor microenvironment could either attract Tregs or induce Tregs [64, 65]. Similar to MDSCs, Tregs also apply multiple mechanisms to broadly suppress the immune cells through contact dependent or independent pathways. Besides IL-10 and TGF-β, the two common factors in the immune suppression mechanism, Tregs could produce other soluble factors to suppress immune cells. For example, galectin-1, a member of β-galactoside-binding proteins, could be released by Tregs as a homodimer and bind to CD45, CD43 and CD7 causing growth arrest, apoptosis and abrogation of proinflammatory cytokine production in activated T cells [66, 67]. Similar to MDSCs, Tregs also compete for cysteine with activated T cells [68, 69], and Tregs compete for IL-2 with the T cells as well [70, 71].

Tregs also apply multiple mechanisms of contact dependent suppression. Tregs constitutively expresses both CTLA-4 [72, 73]and LAG-3[74]. The CTLA-4 could bind to CD80 and CD86 on APCs surface and block the costimulatory signal transduction. The interaction between Tregs and DCs with CTLA-4/CD80 or CD86 could also induce the enzyme indoleamine 2, 3-dioxygenase (IDO) in DCs and induce the immunosuppressive metabolites [75]. The LAG-3 could bind to MHC II on some APCs and are required for the maximal suppressive activity

[76]. Tregs also have a high level of cyclic adenosine monophosphate (cAMP) to maintain their anergic state [77]. By forming gap junctions between Tregs and effector T cells, cAMP in Tregs could diffuse into the T cells and suppress the T cell through the cAMP-protein kinase A type I-C terminal Src kinase inhibitory pathway [76]. Tregs also use perforin/granyme pathway to induce cytolysis of monocytes, DCs, B cells and T cells.

After 100 years of exploring tumor immunology, the complex and dynamic interaction between immune system and tumor development has become clearer. This whole dynamic interaction could clearly be represented by the investigations of genetically modified mouse tumor models. In a mouse model with liver transfection of oncogene Nras through hydrodynamic injection, the expression of Nras could induce the senescence program in those Nras expressing hepatocytes. Immune surveillance was efficiently triggered and started to eradicate the senescent cells 6 days after the oncogene activation through the $CD4^+$ T cell mediated adaptive immune response. 60 days after oncogene activation, all senescent cells were eradicated [78]. In some more aggressive tumor development models, such as oncogene derived transgenic mouse tumor models, both antigen-specific antibodies and T cells were elicited at early stage of tumor development [79-81]. However, the endogenous immune surveillance failed to inhibit the tumor development in any case. Although the titer of the specific antibody increased over time, the specific $CD8^+$ T cells in both tumor host organs and tumor draining lymph nodes were increased at early stages of

tumor development and eventually declined at late stages. Those specific $CD8^+$ T cells were not fully functional and anergic. The T cell tolerance may be induced by the enhanced TGF-β signaling of tumor draining lymph node T cells and increased Tregs in tumors [80].

The endogenous immune surveillance consistently eradicates spontaneous nascent transformed cells through both innate and adaptive immune systems. The occasional tumor initiation event in cell, such as DNA damage and oncogene activation, always first triggers the cellular program to fix the problem or turn off the cell. In the case of oncogene derived tumors, the spontaneous aberrant activation of oncogene could trigger the cellular senescence program and cause cell cycle arrest. The senescence process also induces the cell to start secreting various cytokines and growth factors [82, 83]. This is the time point when the dynamic interaction between immune surveillance and tumor development starts. Depending on the cellular microenvironment, the secreted factors could promote the tumoregenesis, bypass the senescence and escape from the immune surveillance. In most case, with a health immune system, the secreted factors could trigger both the innate and adaptive immune response and efficiently eradicate these senescent cells. However, at some time point in an organism's life, some nascent transformed cells survive by occasional event or/and accumulated factors that impair the immune surveillance, such as virus infection, chronic inflammation and aging [80, 81]. The surviving nascent transformed cells keep evolving under the immune surveillance, and develop multiple mechanisms to

both promote the development and escape the immune surveillance. Eventually, these cells successfully suppress the immune surveillance locally and/or systemically, and establish the primary tumor and metastatic tumors. Interestingly, almost all of the mechanisms that tumors utilize to escape and suppress the immune surveillance are the mechanisms to regulate the healthy immune system of the body or regulate the maternal-fetal interaction during the pregnancy. For example, IDO is released by placenta to prevent immune rejection of fetus and it is also released by tumor cells to suppress the anti-tumor immune responses [84-86]. Some of the mechanisms tumors utilize even are the mechanisms used to inhibit the tumor development at the beginning, such as TGF-β.

In summary, with the 100 years of accumulated knowledge of the tumor immunology, we now much better understand the dynamic interaction between the immune system and tumor development. This reveals the pivotal role of the immune system in the tumor development and also suggests that the proper modulation of the immune system should have great potential to control the tumor development.

1.5 Limitations of Current Therapeutic Cancer Vaccine

Since the original hypothesis of immune surveillance was proposed, people have worked on cancer immunocontrol for more than one hundred years. This is the most clinically tested immunotherapy. Despite the dramatic development of this area, the clinical efficacy of the therapeutic cancer

vaccine is still limited. Only a few therapeutic cancer vaccines are currently available and are restrictive to relatively small groups of cancer patients. The major hindrance is current cancer vaccines could not elicit an efficient immune response to eradicate tumors. With the current understanding of tumor immunology, there are two major causes for this limitation: immune suppression caused by the tumor and the low immunogenicity of the current tumor antigens in the vaccine.

First is the immune suppression of the tumor. The most important criterion for any vaccine development is to induce an efficient immune response. In fact, the occasionally successful cases in those vaccine trials were associated with the relatively higher efficient anti-tumor immune responses directly or indirectly elicited by the vaccine [9, 87, 88]. However, as discussed above, an established tumor already develops a strong immune suppressing microenvironment, as well as the systemic immune suppression. Additionally, most of these clinical trials are in conjunction with other cancer treatments, which could further cause the impairment of their immune system. This is the most important hindrance specific for cancer immunotherapy.

On the other hand, the immunization strategy in these early cancer vaccine trials were not specifically designed to overcome immune suppression factors, instead they were engineered to directly stimulate the immune response through pathways to enhance the antigen presentations, such as using different adjuvants, like GM-CSF and CpGs. Therefore, it is not surprising that most of these early

cancer vaccine trials failed, because of their inability to induce a sufficient anti-tumor immune response, especially the T cell response. The most important reason for the success of the first FDA approved therapeutic cancer vaccine, Sipuleucel-T, is that the immunization strategy of this vaccine partially avoids the immune suppression factors [89, 90]. This vaccine applies the in vitro stimulated DC for the immunization and avoids the immune suppression during antigen processing. 66% of patients with Sipuleucel-T treatment showed an antibody response to the immunized antigen PA2024 and 28.5% showed antibody to prostatic acid phosphatase; and 73% patients and 27.3% patients showed the specific T cell response to these two antigens respectively. The higher antibody activity showed significant association with longer survival time. However, the median increase in survival with the Sipuleucel-T treatment was only 4.5 months. This indicated that without further control of the immune suppression, the therapeutic cancer vaccine still could not elicit meaningful clinical efficacy [89].

The second limitation of the current therapeutic cancer vaccine is the natural immune regulatory system that inhibits the immunogenicity of the antigen components in current vaccine development. The majority of tumor antigens in clinical trials are tumor associated antigens, which are self antigens that are over-expressed in tumors [91-93]. This could be the fundamental hindrance for all of the self antigens as cancer vaccine candidates. The low immune response to tumor antigens, such as breast tumor antigen Her2 and melanoma antigen gp100, has been well documented in both mouse models and clinical studies. For

example, peptide immunization of the T cell epitope $gp100_{209-217}$ with different regimens had a minimal immune response in melanoma patients, as well as in a transgenic mouse model with the most common human MHC class I molecule, HLA-A2 [94-97]. This is the similar situation for breast tumor antigen Her2. Two T cell epitopes derived from Her2 could not elicit high avidity $CD8^+$ T cell in A2/neu transgenic FVB/N mice, comparing to the A2 wide type mice [98]. Further investigations reveal two mechanisms that could cause the low affinity T cell-dominant immune response: the immune tolerance to those self antigens and instability of the peptide-MHC-1 complex. It has been clear that self antigens are naturally tolerant to the immune system. Central immune tolerance deletes most high avidity T cell and B cell against self antigens at thymus and bone marrow during the development. Those high self-avidity T/B cells that escape from central immune tolerance are still under the control of the peripheral tolerance, which are mainly operated by Tregs, as well as other regulatory leukocytes, such as DCs and NK cells. Therefore, the low avidity T cells to these self tumor antigens are dominant in the immune system and more frequently activated by immunization. The immunization of the NY-ESO-1, the cancer-testis antigen, elicited a high frequency of low affinity T cells in the ovarian cancer patients [99-101]. However, the high avidity T cells could be detected by in vitro stimulation without Tregs [102]. This indicated the low frequency and high avidity T cells are tightly controlled by the peripheral tolerance.

The instability of peptide-MHC-1 complex is another possible mechanism responsible for the low immune response. The instability of the peptide-MHC-1 complex could inefficiently present the T cell epitope to naïve T cells, therefore could inhibiting the eliciting of a strong immune response. The evidence for this mechanism comes from the observation of the low immune response elicited by the immunization of the $gp100_{209\text{-}217}$ in the A2 transgenic mice whose $gp100_{209\text{-}217}$ was knocked out [96]. And faster rate of the dissociation of the epitope from the HLA-A*0201 was also confirmed through the biological and physical analysis. In any case, these self tumor antigens are not good immunogenic antigens.

In summary, the inefficient immune response has been to date characteristic of the therapeutic cancer vaccine. Without strong antitumor immune responses, the therapeutic cancer vaccine could not achieve the sufficient clinical efficacy. However, with additional methods, such as combined treatment with immune modulators and modification of the antigens, the therapeutic cancer vaccine may overcome this hindrance. On the other hand, because most tumor antigens are self antigens, the risk of the autoimmune disease will increase with the improvement of the antitumor immune responses to these self antigens. With this catch-22, the contribution of the therapeutic cancer vaccine with self antigens may be limited for cancer control. Using tumor specific antigens for therapeutic vaccines may overcome the problem of auto-immunity and low avidity T cells, but would still be restricted by immune suppression initiated by tumor at early stages.

1.6 Cancer Immunoprevention

Although cancer immunotherapy is the most tested strategy of cancer immunocontrol, cancer immunoprevention is the original suggestion of the cancer immune surveillance theory: Elicit efficient immune response to eliminate the nascent transformed cells and then eradicate the cancer. Mainly because of practical reasons, the strategy of cancer immunoprevention was neglected for a long time. The successes of the HPV and HBV vaccines in prevention of the related cancers, as well as the better understanding of tumor immunology, the idea of cancer immunoprevention is becoming more attractive. Especially, with the effort of Dr. Johnston and other initiatives, the National Breast Cancer Initiative has started the Artemis Project to develop a prophylactic breast cancer vaccine to prevent breast cancer.

To date, the concept of cancer immunoprevention has been developed and replenished by multiples strategies. There are two major types of strategies: 1) removing the cancer risk factors, such as HPV and HBV vaccines and 2) eradicating nascent transformed cells through immunoprevention, such as prophylactic cancer vaccine. The fundamental difference between these two strategies is the targets. The first strategy targets the cancer risk factors and prevents initiation of the tumor cells. The second strategy targets the nascent transformed cells and prevents the further development of the initiated tumor cells [103]. These two strategies are not exclusive to each other. The combination of the two strategies may have better efficiency in preventing cancer.

The first strategy has been partially realized with the development of the vaccines against cancer related viruses, such as HBV and HPV. Vaccines developed against cancer related infections have the potential to prevent about 17.8% of cancer, which is the estimated percentage of the pathogen infection related cancer. Another part of this strategy is controlling chronic inflammation, which has been recently confirmed as one of the major risk factors of tumor initiation and development [104]. This could also be accomplished by chemical drugs, such as aspirin.

The second strategy was directly suggested by the original concept of the cancer immune surveillance. However, all of the achievements in this area were still mainly limited in the animal models. The current strategies for this immunoprevention area are almost totally adopted from the cancer immunotherapy. This could be classified into two types: 1) prophylactic cancer vaccines that target tumor antigens; 2) enhancement of immune system by immune stimulators and immune modulators. However, some limitations of cancer immunotherapy also could be directly adopted by the immunoprevention, and even be enlarged, such as the risk of the autoimmune diseases.

1.7 Prophylactic Cancer Vaccine

1.7.1 Introduction

The cancer vaccine was first inspired from the success of infectious disease vaccines, and then mainly focused on the development on the therapeutic

cancer vaccine to treat the cancer patient. However, there is no infectious disease vaccine, which efficiently controls the established infections alone. This is the same in the therapeutic cancer vaccine development. The prophylactic cancer vaccine gained popularity after the successes of the HBV and HPV vaccines in the prevention of related cancers. The concept of the prophylactic cancer vaccine is as simple and straight forward as the infectious disease vaccines: eliciting a sufficient and specific anti-tumor immune response in healthy people and eradicating nascent transformed cells whenever they emerge during the organism lifetime preventing cancer.

It has been well clarified that the nascent transformed cells are completely different from the established tumor cells at all levels: genome, transcriptome and proteome. A nascent transformed cell needs to evolve in all these levels, and adopt the characteristic ten hall marks to become a fully developed tumor [105, 106]. The tumors are very heterogeneous. There is no known cancer deriving genes found in all cancers, even in cancers in the same organ. For example, the $Her2^{+}$ breast cancer only represents about 25% breast cancers. Recent deep tumor sequencing studies revealed that heterogeneity is typical in all types of tumors. Different tumor cells in the same tumor differ in mutation profile, which is accumulated as the tumor evolves under the different selecting suppression, such as growth suppression and immune suppression [107]. The evolution of tumor cells creates moving targets for the therapeutic cancer vaccine and fundamentally causes the inefficiency of these vaccine trials. The lesson learned from the

failures of the therapeutic cancer vaccine suggests targeting the nascent transformed cells is a much more efficient way to prevent tumors.

However, most current strategies in prophylactic cancer vaccine development are directly adopted from the therapeutic vaccines, as well as the limitations of the therapeutic cancer vaccines [103, 108-112]. Some of these limitations are decreased in the prophylactic setting, such as efficiency of eliciting immune response, while some are amplified, such as the risk of the autoimmune diseases. I will discuss some major advantages and disadvantages of current strategies for prophylactic cancer vaccine development compared to therapeutic cancer vaccines.

1.7.2 Advantages in Eliciting Efficient Immune Response

As discussed above, the biggest hindrance of the cancer immunotherapy is the immune suppression derived by the tumor or natural immune tolerance. This could inhibit the vaccine to induce a strong anti-tumor immune response. Compared to the therapeutic cancer vaccine, the main advantage of the prophylactic cancer vaccine is there is no tumor derived immune suppression in healthy people. Without the immune suppression, the prophylactic cancer vaccine could efficiently elicit strong immune responses. With advanced vaccine development strategies, both T cell and B cell immune responses against current tumor antigens could be robustly elicited in a variety of mouse models as well as in the clinical cancer vaccine trials. Even in the presence of tumor derived immune suppression, more than 50% of the immunized cancer patients still

could successfully elicit an immune responses against the vaccine antigens in the trial with Sipuleucel-T [89]. The natural immune tolerance to those self tumor antigens also could be overcome with different strategies, such as epitope modifications. For example, in transgenic mouse models which have the natural immune tolerance to the $gp100_{209\text{-}217}$, the immunization of the natural epitope could not elicit the high avidity T cells. However, the strong immune response with high avidity T cell stimulation was achieved by the epitope modifications of the $gp100_{209\text{-}217}$ [96]. Other advanced immunization platforms also could break the natural immune tolerance, such as strong adjuvant and immune modulators.

Tumor derived immune suppression could not only suppress the stimulation of efficient immune responses, but also could reduce the activated immune cells in the tumor derived microenvironment. The impaired immune response accelerates the evolution of tumor cells and their ability to escape from immune surveillance by altering the antigen presentation pathway. Different from the therapeutic vaccine, the prophylactic cancer vaccine targets the nascent transformed cells. These cells do not have the established immune suppression microenvironment as do tumors at later stages. First, the factors, such as TGF-β, that tumors utilize to suppress immune responses are the same factors for the efficient immune surveillance at the beginning. The function of TGF-β to promote or suppress the immune surveillance is determined by the context of the environment [80]. Second, the endogenous immune surveillance has a lag in the switch from innate immune response to more specific and stronger adaptive

immune response. This lag offers the time window for nascent transformed cells to further evolve and establish the immune suppression context [81, 82]. The pre-activated adaptive immune response by the prophylactic immunization avoids the latency of the natural immune response switch and could more efficiently eradicate the nascent transformed cells [81, 113]. This leaves no chance for nascent transformed cells to further evolve and develop all kinds of mechanisms for survival.

The prevention efficacy of prophylactic immunizations has been proven in numerous mouse tumor models with genetic or carcinogen-induced cancer risk, including the BALB-NeuT mice, one of the most aggressive mouse tumor models [113-118]. This transgenic mouse model has active rat Her2/neu oncogene expressed under the mouse mammary tumor virus promoter. All of the ten mammary glands develop breast tumors in 30 weeks [119, 120]. With the proper prophylactic immunization of Her2 that started from 6 weeks old, BALB-NeuT mice could keep palpable tumor free up to 2 years [113]. The gene transcriptome profiles of mammary glands from these tumor free mice at 15 weeks old and 26 weeks old were clustered with the 6 weeks old non-treated BALB-NeuT mice [115]. This demonstrated the efficient prophylactic cancer vaccine could prevent the nascent transformed cells from further developing and evolving. In a natural setting, the tumor initiation events are spontaneous and even the endogenous immune surveillance can efficiently eradicate most of them.

1.7.3 Disadvantages of Autoimmune Disease Risk

Most of current tumor antigens are tumor associated antigens (TAAs), which are abnormally over-expressed in tumor cells and down-regulated in normal adult cells [103, 108]. Basically, the immune responses elicited by these TAAs areself-antigen responses, which are the risk factors for autoimmune disease. Although these TAAs were well tolerated in both animal models and clinical trials [121]; eventually, the autoimmune disease risk of current therapeutic cancer vaccine needs to be addressed. However, with the fact of poor survival rate with the therapeutic cancer vaccine treatment, this is not the current priority. This is not the situation for the prophylactic cancer vaccine, whose recipients are healthy populations. This is the fundamental disadvantages for the prophylactic cancer vaccine based on these TAAs.

Another problem for the prophylactic cancer vaccine based on TAAs is that most TAAs are tumor type specific. For example, $Her2^{+}$ positive breast cancer only represents about 20% to 25% of the breast cancer. There is no way to accurately predict the cancer type of the potential patient. This limits the subsector of this cancer type specific prophylactic cancer vaccine could be vaccinated

In summary, the prophylactic cancer vaccine is becoming an increasingly popular strategy for cancer prevention, and has started to attract more research attention. Current prophylactic cancer vaccine development mostly follows the strategy for the development of therapeutic cancer vaccine. Compared to the

therapeutic cancer vaccine in cancer control, the prophylactic cancer vaccine has both significant advantages and disadvantages. The prophylactic cancer vaccine could robustly elicit specific immune responses with the healthy immune system and could more efficiently eradicate the target cells, since they have not set up the established immune suppression. This advantage is based on the prophylactic immunization, not after tumor initiation as currently done.

However, because the prophylactic cancer vaccine needs to cover a longer lifespan than the therapeutic vaccine, the prophylactic cancer vaccine has more risk for autoimmune disease with the current cancer vaccine candidates. Additionally, without knowing the specific risk of the cancer type in normal populations, the design of a prophylactic cancer vaccine is more complex and limited with current vaccine antigen candidates.

All of these indicate that the prophylactic cancer vaccine is different from the therapeutic cancer vaccine. Therefore, it is necessary to develop a strategy that is suitable for the prophylactic cancer vaccine development.

1.7.4 Discussion of strategy for prophylactic cancer vaccine development

The targets of the prophylactic cancer vaccine are the potential transformed cells and the nascent transformed cells. Vaccines against cancer related viruses, such as HBV and HPV, could eradicate the virus infected cells that have the potential to transform. About 17.8% of cancers are proposed to be related to the pathogen infections [13]. However, there are only two types of human cancer related viruses that have been confirmed so far: HBV and HPV.

Their related cancers have been controlled by the anti-virus vaccines. Here, I will focus on the nascent transformed cells. This is the area we do not have any breakthrough to clinical application yet. Since the prophylactic cancer vaccine targets nascent transformed cells, it would also include the virus-induced transformed cells.

The targets of the prophylactic cancer vaccine determine what the vaccine candidates should contain. There are two pivotal characteristics: 1) expression in nascent transformed cells and 2) wide coverage of different types of cancer. Identifying the early expression antigens in nascent transformed cells is the most critical step for the prophylactic cancer vaccine. With current advanced immunization technologies, the stimulation of a strong immune response is not the biggest hurdle for developing a prophylactic vaccine. The correct targets of nascent transformed cells fundamentally determine if the vaccine could efficiently eradicate the tumor cells in the first place, where the endogenous immune surveillance starts losing the whole war against cancers.

The evolution of the tumor makes it a moving target. This causes most tumor antigen screening efforts to focus on the cancer deriving antigens which could be the constant references for the anti-tumor responses. However, those tumor antigens are not necessarily expressed in the nascent transformed cells. For example, the mutation of ras gene was detected in 58% of colon adenomas larger than 1 cm and 47% of carcinomas, while there were only 9% adenomas less than 1 cm that carried the ras gene mutations [122]. This will make ras-mutant based

vaccine essentially a therapeutic vaccine for these patients, even if it was immunized prophylactically.

Most cancers need a long time to develop from the nascent transformed cells to the established tumor cells. Even in the people with inherited gene mutations, it still takes decades for them to eventually develop cancers. For example, in Lynch Syndrome patients, who carry mutant genes of the DNA mismatch repair pathway, 80% of patient will not develop colon cancer until after 44 years of age[123]. Additionally, as I pointed out previously, one of the advantages of the prophylactic immunization is efficiently preventing further evolution of the nascent transformed cells. Therefore, the nascent transformed cells are relatively quiescent targets for the prophylactic cancer vaccine. This suggests that all of the alternations of the nascent transformed cells are the potential vaccine candidates, as long as they can present properly to the immune system and are specific from the normal cells. This could include genetic mutations, alternative splicings and post-translational modifications, even if it is not integral to tumor development.

Another essential criterion for the prophylactic cancer vaccine is the wide coverage of tumor types. Most cancers are unpredictable with current knowledge and techniques. Even the cancer from the same organ, such as mammary gland, could be divided into multiple subtypes based on the histological and transcriptome differences. Without knowing the specific cancer risk of most healthy people, an efficient prophylactic cancer vaccine needs to cover different

cancer types. This suggests we need to screen the common alterations among the different nascent transformed cells. Most of cancer researches focus on characterizing the advanced cancers and has repeatedly shown that heterogeneity is a typical characteristic of advanced cancers, which was discovered at the 1950s [124-126]. It is now widely accepted that the heterogeneity of the tumor is mainly caused by tumor evolution [127]. This indicates that for each tumor, it is more homogeneous at early stages. However, there is little research on characterizing the alterations of different nascent transformed cells. We hypothesize that there are common alterations in different nascent transformed cells. First, genome instability affects all the levels of the cellular functions, and generates both "driver" alterations and "passenger" alterations [128]. As mentioned before, both specific alterations could be the candidates for the prophylactic cancer vaccine. This indicates it may be possible to find the common alterations for vaccine development. For example, we have discovered that FS transcripts are whole category of alterations that are frequently detected in different cancers [129]. Second, we also have discovered that cancer patients have common antibody profiles through the immunosignature analysis, and those profiles are distinguished from the healthy people [130]. We also can detect the common immunosignature changes of the early tumor stages in the mouse tumor models (unpublished data). The immune response reflects both historical and current exposure of the antigens. The common immunosignature of cancers indicates there are common immunological alterations in these different cancers, even at

early stages. Those common immunological alterations can be the candidates for the prophylactic cancer vaccines. We should explore more the characterizing of alterations in nascent transformed cells in the future for the development of prophylactic cancer vaccine.

In summary, the prophylactic cancer vaccine is an old concept but needs a new strategy for further development. I have discussed two essential characteristics of vaccine antigens for prophylactic cancer vaccine development by comparing it to therapeutic vaccines. Besides these two, the specificity of the antigens to tumor cells is also critical for the antigen screen to minimize the risk of inducing autoimmune disease. However, tumor antigen screens in nascent transformed cells have received little attention in current cancer research. With the development of high throughput research technologies, such as deep sequencing, more common and specific antigens in different nascent transformed cells will be discovered.

1.7.5 Prophylactic cancer vaccine and metastasis cancer vaccine based on the Frame Shifted antigens

Our overall goal is to develop a broad-spectrum prophylactic cancer vaccine to prevent a variety of different cancers. We are systematically applying bioinformatics, proteomics, and immunological methods to achieve this vision. I will discuss a novel strategy for the prophylactic cancer vaccine development, which is developed in our lab. One of our foci toward developing the prophylactic cancer vaccine is the identification of frame shifted (FS) neo-peptides in

nascent tumor cells as vaccine components. FS peptides are derived from transcripts expressed in alternate reading frames; these are activated by various gene mutations and abnormal splicing events in tumor cells. Based on the criteria I discussed above for the prophylactic cancer vaccine antigens, there are several advantages of the FS antigens.

First, FS antigens can efficiently elicit a specific immune response with proper administration. A prophylactic cancer vaccine would be administered to a healthy population with a competent immune system. Unlike a therapeutic cancer vaccine, it doesn't need to overcome any immunosuppression caused by an established tumor. Additionally, FS peptides come from mutant proteins and are encoded by the 2^{nd} or 3^{rd} reading frame of corresponding regions of genes. Consequently they should be seen as foreign antigens without natural tolerance of the immune system. These FS antigens can elicit high affinity antibodies and T cells more easily compared to current self tumor antigens.

Second, FS antigens are an abundant source for the screen. The tumor is developed stepwise from nascent transformed cells. As summarized by Dr. Hanahan and Dr. Weinberg, nascent transformed cells need to achieve ten common hallmarks to become tumors, such as sustaining proliferative signaling, evading growth suppressors, resisting cell death, activating invasion, metastasis and so on [105, 106]. Among all of the ten hallmarks, the genome instability is the molecular foundation for a nascent transformed cell developing all other hallmarks [106]. In addition to activating distinct driving factors for different

tumor development, genome instability also affects other factors which are not necessarily associated with tumor development, including genes, transcriptomes, and translated proteins. These aberrant features are sources for the generation of FS antigens. Because of this global affect genome and transcriptome instability, we believe the FS antigens are more commonly shared across all kinds of nascent transformed cells and tumors. For example, SMC1A FS has been detected in almost all of the tumors we have screened so far. While most of the tumor associated self antigens on the other hand are only shared in distinct tumors.

Third, the FS antigens are specific to tumors. Risk of autoimmune disease is a concern in both therapeutic and prophylactic cancer vaccine development. Especially for the prophylactic cancer vaccine, since it is applied in healthy people and needs to be activated for a much longer time than the therapeutic vaccine. The specificity of a tumor antigen has several levels: gene level, transcriptome level and protein level. The specificity of the FS antigens is significant in all of these three levels.

First, the stability of the genome in normal cells decreases the chance for templates of FS transcripts and the production of FS transcripts by mis-functional transcription factors. Tumor cells have higher gene mutation rates compared to normal cells as well as a higher incidence of translocation [131-133]. High gene mutation rates cause high levels of FS transcripts, especially in those tumors with high levels of microsatellite instability (MIS). These microsatellite regions are sensitive to genome instability caused by defective DNA mismatch repair. We did

bioinformatics analysis of tumor sequence data shows that over 98% of mutations in microsatellite regions are FS mutations [129].

The stability of the genome in normal cells also secures the normal function of gene duplication and transcription machinery at the fundamental level. Many transcription factors change during tumor initiation and development. These changes are caused either by direct mutations and abnormal expression of these factors or by the change of upstream signaling factors. For example, over 40% of alternative splicing events are significantly changed in breast and ovarian cancer tissue compared with normal breast and ovarian tissues. And other studies estimated that 35% of the alternative splicing could produce a FS transcript [134, 135].

Second, the quality of the transcriptome in normal cells is tightly controlled by multiple mechanisms, especially by nonsense mediated decay (NMD) which targets most transcripts that contain premature termination codons (PTCs). The normal function of NMD could protect people carrying a PTC allele and prevent autosomal recessive disorders. For example, patients with a PTC containing β-globin allele usual are phenotypically normal, because NDM eliminated most PTC containing transcripts. However, some PTC containng β-globin transcripts are insensitive to NDM. As a consequence, high level truncated β-globin is expressed and causes anemia [136]. Most FS transcripts contain PTCs, and they are favorite targets of NMD. About 90% of PTC containing transcripts in normal cells are efficiently eliminated by NMD[137] . This process would make it

unlikely a FS transcript would produce a FS peptide in normal cells. Although the activity of the NMD in tumor cells is still controversial, the up regulation of the FS transcripts by the genome instability still causes more FS transcripts escaping from the NMD [138].

Third, besides these two control systems, normal cells have another fully functioning quality control system: endoplasmic reticulum associated degradation (ERAD) [139]. Truncated proteins with C-terminal FS peptides cannot be processed correctly and properly folded. Those truncated proteins produced from leaky FS transcripts in normal cells will be efficiently detected and destroyed by ERAD [140]. Therefore even if some FS transcripts escape from NMD, they will be destroyed before being presented on the normal cell surface. With all these security controls, normal cells will efficiently avoid attack from a specific immune response to FS antigens.

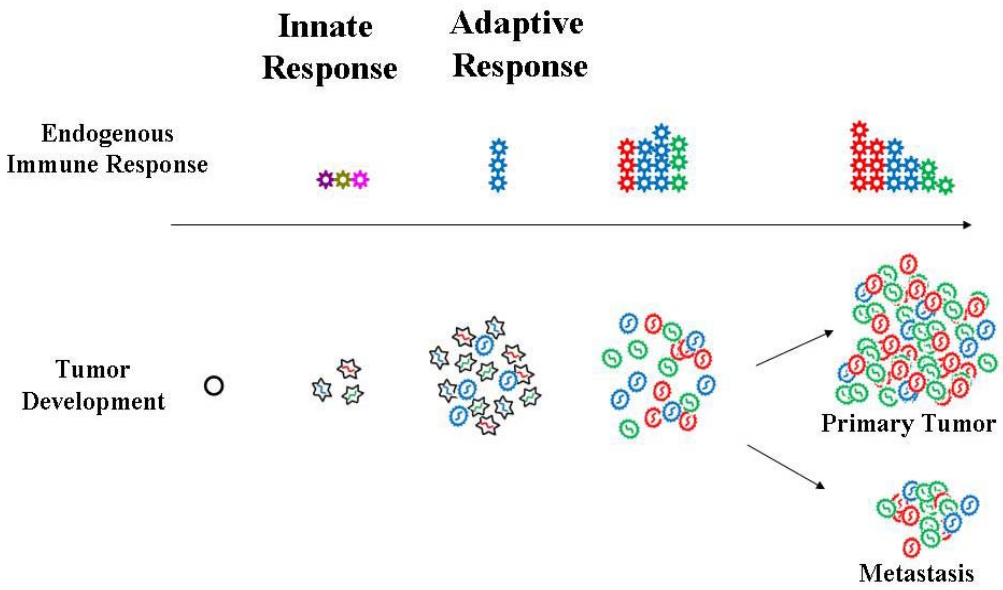

Figure1. 1 Model of FS antigen presentation during tumor development

All of the characteristics discussed above make FS antigens good candidates for prophylactic cancer vaccine development. According to the research from my years of study, I developed a model of FS antigen exposure to the immune system during tumor development (Figure 1.1). It is a simplified model to emphasize the FS antigen exposure and development of endogenous immune response to those antigens. The FS transcript starts to accumulate right after transformation is initiated in normal cells by different factors such as viral infection, oncogene activation, and exposure to carcinogens. At this earliest stage, the nascent transformed cell still maintains normal functions of quality control systems to prevent the presenting of these FS antigens. As a tumor keeps developing, the gene mutations increase and normal cellular functions become deficient. The FS antigens start to leak from the quality control systems and can be presented on the tumor cell surface. Meanwhile, maybe earlier, apoptosis

and senescence are trigged in some tumor cells and different cytokines and chemokines are released, which attract immune cells and induce the endogenous innate and adaptive immune response. Presumably in most cases this early immune response is effective in eliminating these cells. However, if not, the tumor keeps evolving under immune surveillance and develops mechanisms to suppress anti-tumor immune response. Although more and more FS antigens are exposed to immune system during tumor development, the endogenous adaptive anti-tumor immune responses are more suppressed, including the FS immune responses. The tumor metastasis starts at the early stage of tumor development; those clones still share some common FS antigens with the primary tumor. By this time, immune suppression is getting stronger; the micro-metastasis tumors are developing faster. It usually takes 5 to 50 years for a nascent transformed cell to develop into a malignant tumor and develop metastatic tumors, especially in a developing solid tumor [141, 142]. Without any stimulation, the endogenous anti-tumor immune response develops slowly and eventually gets suppressed.

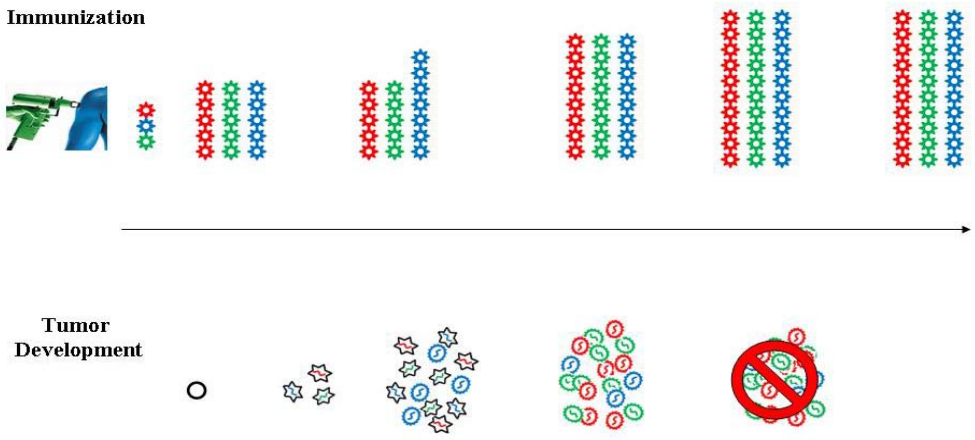

Figure1. 2 Strategy of prophylactic cancer vaccine based on FS antigens

According to this model, I developed a strategy for prophylactic vaccine development (Figure 1.2). With the active anti-tumor immune response, the immune system could efficiently detect and destroy the nascent transformed tumor cells without the established immune suppression induced by a developed tumor. There are two important characteristics for an efficient prophylactic cancer vaccine: First, the vaccine should include antigens that start to present at the early stage of tumor development. It will let the immune response catch the nascent transformed cells. The lessons from different vaccine trials indicate that completely controlling different tumor developments with a single antigen is difficult. Additionally, with the limitations of current studies, we cannot predict which antigens will present first. The goal is to include a pool of FS antigens in our prophylactic cancer vaccine based on the frequency analysis of genetics in a variety of tumors as well as the protection analysis of each antigen in different models.

Secondly, an efficient prophylactic vaccine should be administrated early in healthy individuals and elicit a proper immune response. For a prophylactic cancer vaccine, another important factor is to maintain a relatively strong immune response and an efficient memory immune response, especially to those antigens which present in late stages. These factors rely on the immunization regimen and an efficient adjuvant. In the mouse model, we developed a method to efficiently deliver both big (such as plasmids) and small (such as CpGs) nucleic acid fragments through a genetic immunization with a gene gun [143-146]. We are also developing methods to efficiently deliver other formats of adjuvants, such as siRNA and proteins. All of these developments facilitate the screening of vaccine candidates.

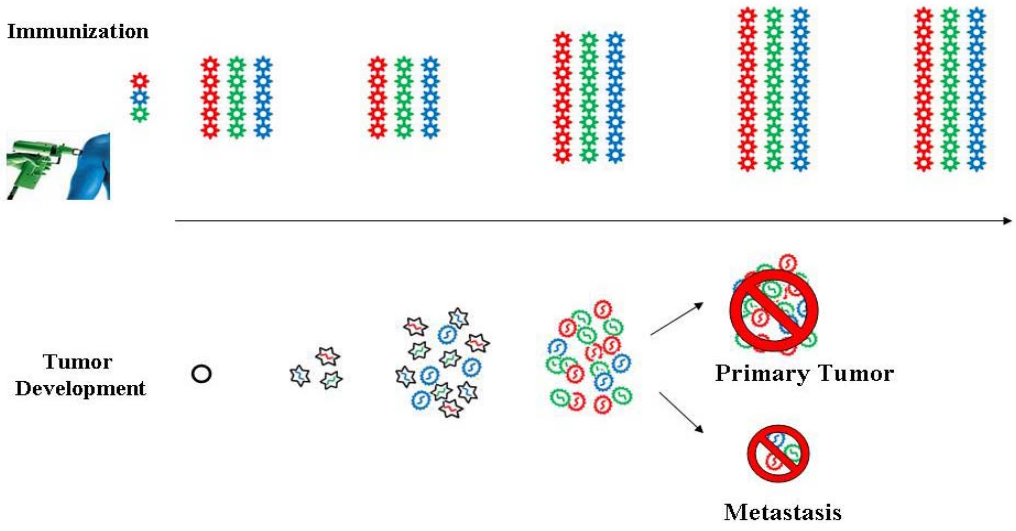

Figure1. 3 Strategy of prophylactic cancer metastasis vaccine

Based on the concept of a prophylactic cancer vaccine against the primary tumor, we also developed a sub-project to develop a prophylactic metastasis

cancer vaccine (Figure 1.3). Control of metastasis has an important clinical application in cancer treatment. 90% of the deaths caused by cancer are due to metastatic tumors [1, 3]. Most primary tumors on the other hand, especially solid tumors, are treatable. Evidence shows that tumor metastasis can start at an early stage of tumor development, even before any clinical symptoms of cancer [147-149]. The early micro metastatic clones are not detectable with current technology and usually are resistant to current drugs. They usually take a long time to develop into a metastatic tumor. Because of the inherited genomic instability, these micro metastasis tumors still could share a similar gene expression profile the primary tumors [150-153]. Therefore, the prophylactic cancer vaccine containing those shared antigens should also be able to eradicate tumor metastasis. Our data demonstrates that the development of the primary tumor will also boost the specific anti-tumor immune response elicited by prophylactic vaccination. It enhances the immune system to destroy those micro metastasis tumors. Our strategy of the prophylactic cancer vaccine could prevent both primary tumor and metastatic tumor.

1.8 Preventing tumor development by innate stimulation of the immune system

The research of interaction between the immune system and tumor development shows that the innate immune system is also playing an important role in tumor development. The innate immune system can inhibit tumor development; meanwhile, the improper innate immune response also can

promote tumor development, such as chronic inflammation can lead to a tumor. Tumor cells can also recruit innate immune cells to escape immune surveillance, such as MDSCs and TAMs. These facts indicate that a properly active innate immune response could be the first defense to prevent tumor development. Studies of different innate immune system stimulators exhibit impressive effects on tumor prevention in various animal tumor models as well as in clinical trials and treatments of various cancers [153-156]. Most infectious diseases can elicit an efficient innate immune response. The remission of malignant diseases caused by infection was first observed in the 18th century. In the 1890s, Dr. William Coley found that a bacterial infection could induce cancer regression after sarcoma surgery and developed the first immunotherapy formula for cancer named "Coley Toxins". Another innate stimulator, Mycobacterium bovis bacillus Calmette-Gerin (BCG) was also found to have antitumor activity against a variety of cancers such as colon, lung, bladder, and skin [154, 156, 157]. The BCG is now a widely accepted clinical treatment in cancer immunotherapy, especially in the treatment of superficial urothelial carcinoma. Various effective components of BCG are identified for its antitumor activity such as polysaccharides, lipids, and other protein antigens that are irrelevant to tumors. The most important anti-tumor component that was identified was CpG ODN, which is now widely used in the treatment of both infectious diseases and cancers [158-160]. In addition to bacterial infection, other infections, such as virus infections, also exhibit anti-tumor activity. The exact mechanisms of the anti-tumor activity of different

components by innate stimulation are variable. The most important mechanism is the active innate immune response through different Toll-like receptors (TLRs), which are extensively characterized pattern recognition receptor families for factors associated with bacteria, fungi or viruses.

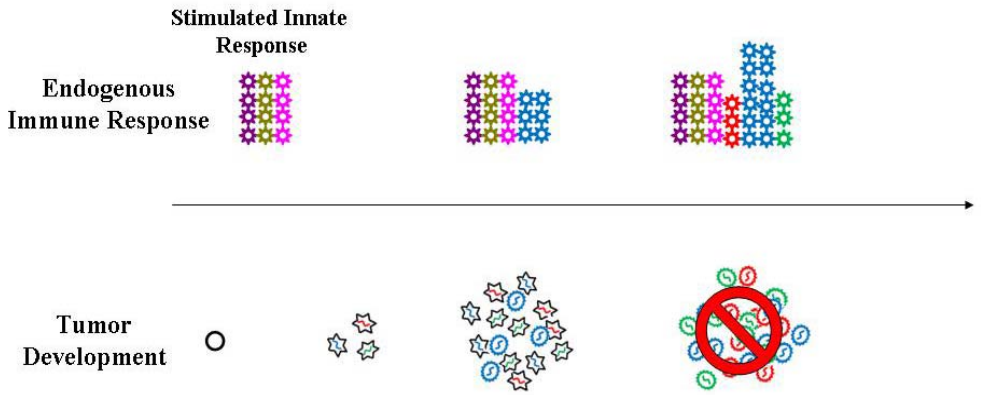

Figure1. 4 Strategy of innate stimulation to prevent tumor development

Similar to cancer vaccine development, most current studies and trials of anti-tumor innate stimulation are focused on cancer treatment. We proposed here to develop a system of sustained stimulation of the innate system to prevent tumor development (Figure 1.4). The interaction between the immune system and tumor development starts at the beginning of the transformation process of a normal cell. The initiation of transformation of a normal cell, such as aberrant expression of oncogenes, could induce cellular senescence or apoptosis. The factors released by these cells first trigger the innate immune response and could have pro- and/or anti-tumor effects depending on the context of the environment. For the model of FS antigen presentation that we proposed above, our strategy of a prophylactic cancer vaccine works at the stage when the adaptive immune response is

involved in the interaction. The strategy of innate stimulation could allow us to prevent tumor development at an earlier stage by directing the innate immune response to exhibit anti-tumor effects. Consequently, the active anti-tumor innate immune response could also enhance the adaptive immune response against tumor development.

Although the current treatment of innate stimulation yields impressive effectiveness, the side effects of these treatments are still the major concern in the clinic. For instance, mild cystitis is common in about 90% of the patients who receive the BCG treatment. The severe side effects of the treatment can cause death, although this occurs in less than 5% of the patients [161]. These results indicate that it is necessary to reduce the toxicity of the treatment, especially for our strategy of prophylactic innate stimulation. The identification of specific effective components is a good direction of the effort since they are much easier to characterize and administer. Therefore, it will be easier to control the side effects, such as toxicity.

Study of the B2L protein which was identified from *parapoxvirus ovis* in our Center shows that this protein can inhibit tumor growth in the transplant mouse tumor models without a detectable specific immune response to the B2L protein [162]. Results of my prophylactic cancer vaccine studies indicate CpG and GMCSF also have the potential to prevent tumor development. These kinds of innate stimulators are good components for cancer prevention.

1.9 Cancer Detection

Early cancer diagnosis is another important aspect in cancer prevention. Most caners are curable in early stage with current therapies. For example, 5 year survival of early stage ovarian cancer is up to 95%, while it is only 25% to 30% in late stage patients [163]. The main areas of focus for early cancer diagnosis are detection through imaging and serological diagnostics. Although imaging technology has had tremendous advancements in the last decades, it is still not sensitive enough for early cancer detection or not applicable for routine screening for various cancers during physical examinations. Serological diagnosis is ideal for early cancer detection, since blood travels around all parts of the body and carries the information of each organ.

Actually, all components found in tumors can be detected in the blood of cancer patient, such as cancer associated antigens, autoantibodies against cancer associated antigens and cancer associated nucleotides, such as RNA and DNA. These three different types of biomarkers are the main focus for early cancer diagnostic development in the serological analysis. The proteomics analysis of the tumor associated antigens attempts to directly detect these biomarkers in the blood. Those tumor associated antigens are usually released by the tumor. With the remarkable progress of proteomic analysis technology, such as mass spectrometry, the sensitivity of proteomics analysis has increased dramatically.

However, the sensitivity of proteomic analysis is limited by the biomarker secretion rate of the tumor and the dilution factor of the volume of whole blood.

For example, in the prostate-specific antigen (PSA) test for prostate cancer diagnosis, Amelie et. al. estimated the minimum detectable prostate tumor size with current PSA analysis ranged from 27 mm^3 to $3.45x10^5$ mm^3. [164]. Similar to the PSA detection, Hori et. al. estimated that with the current serum CA125 assay for ovarian cancer detection, the detected tumor size will be 25.36mm and it will take about 10 years for the tumor to develop to that size [165]. Even with the ideal tumor specific proteomic biomarker, it still needs 8.8 years before the tumor can be detected at the size of 10.52mm [165]. This is not a sensitive enough test for early cancer diagnosis

Unlike proteomic analysis, the signal of both autoantibodies and nucleotide biomarkers can be more sensitive by signal amplification through different pathways. For the autoantibody biomarker, the signal is endogenously amplified by the immune system. The active B cells can dramatically amplify the signal of antigen stimulation by secreting antibodies. The signal of the antibody could also be amplified in vitro by using enzyme-linked immunosorbent assay (ELISA), protein/peptide micro-arrays, or other antibody detection technologies. For the nucleotide biomarkers, the signal can be robustly amplified by in vitro methods, such as polymerase chain reaction (PCR). The sensitivity of these biomarker detections could be improved by the technology developments such as equipment upgrade, in vivo or in vitro signal amplification.

However the specificity of the biomarker detections is not usually directly proportional with the sensitivity improvement. The specificity of those

biomarkers is limited by their own characteristics. Most of these types of biomarkers are not specific to tumors; they are either overexpressed in the tumor cells or induced by the overexpressed targets in the tumor cells. The determination of the over-expression of a biomarker is so variable in the population and could be effect by other diseases. For example, the current ovarian cancer serum biomarker CA125 can be induced by benign gynecologic causes, such as pelvic inflammatory disease, and nongyneologic disorders, such as peritoneal inflammatory disorders [166]. The CA125 serum level above the current ovarian cancer threshold of 35U/ml may be detected in 1% of the healthy population and 6% of the patient with benign disease [167]. For example, normal cells also express PSA, a current biomarker for prostate cancer. The false positive of the prostate cancer diagnosis based on PSA detection is approximately 80%. The nucleotide biomarkers are similar to proteomic biomarkers. For example, most micro RNA biomarkers have comparably higher levels in cancer patient blood compared to normal people. The specificity limitation of the microRNA biomarkers is the sensitive quantification of the in vitro signal amplification without a reliable internal reference. One of the specificity limitations of the autoantibody is that some of those are shared biomarkers with other diseases. For example, autoantibody against c-myc is also detected in autoimmune disease, such as systemic lupus erythematosus [168].

These limitations of current serological biomarkers indicate that it is necessary to discover more specific biomarkers with robust detection methods.

CHAPTER 2

PROPHYLACTIC CANCER VACCINE DEVELOPMENT

2.1 Introduction

Cancer prevention through the prophylactic cancer vaccine is a historical goal of the cancer research area and it has been revised recently with the development of an approved therapeutic cancer vaccine. However, most recent strategies for prophylactic cancer vaccine development are still limited by the vaccine antigens that have been tried for the therapeutic vaccines. We summarized previously studies and developed our own strategy of the cancer prophylactic cancer vaccine development, which is based on the FS antigens.

The inherited characteristics of genomic instability in tumor cells make FS antigens a good source of candidates for screening for prophylactic cancer vaccine development. The FS transcript can be generated by various processes that are fundamentally affected by genomic instability, such as abnormal alternative splicing, trans-splicing, translocation and microsatellite instability (MSI).

Particularly, MSI can be caused by genetic FS mutations in genes containing coding microsatellites and are well documented in colorectal, gastric and endometrial cancers with DNA mismatch repair deficiency. For example, by analyzing 11 MSI-high colon cancer cell lines, David *et. al* showed high FS mutation frequency of genes containing coding microsatellites, including 13 previously reported genes and 5 new candidates [169].

Abnormal splicing is another source of FS transcripts. The tumor associated and specific abnormal splicing events are also well studied, while most of them focus on the functional analysis of these splicing events for tumor development. By bioinformatics analysis of the Expressed Sequence Taq (EST) database, we detected 43 tumor specific and 53 tumor associated FS transcripts that encode longer than 8 amino acids FS peptides in various tumor EST libraries [129].

In addition to those two well studied sources, we also discovered a de novo source of FS transcripts: the chimeric FS transcripts which contain two different genes with a shifted reading frame of downstream gene. These transcripts can be caused by translocation at the genomic level or trans-splicing at transcriptional level. After years of study, we generated a list of the chimeric FS candidates by bioinformatics analysis and cDNA sequence confirmation, including 48 chimeric FS transcripts that were validated in 50 human breast tumor cell lines and 68 primary human breast tumors. All of these FS antigens are suitable candidates for vaccine development. *In silico* analysis reveals that the whole list of FS candidates contains enough epitopes to cover most HLA haplotypes in the human population. Although different types of tumors possess different FS transcript profiles, the whole list of FS candidates is enough to cover the tumor types that we have investigated so far.

We tested our concept of a prophylactic cancer vaccine in two types of mouse tumor models: a transplant mouse tumor model and a transgenic mouse

tumor model. In the transplant mouse tumor model, we set up the mouse breast tumor model 4T1/Balb/c and the mouse melanoma tumor model B16F10/C57BL6. These models allowed us to quickly evaluate FS candidates. We also set up two strains of transgenic mouse models which were BALB-NeuT and FVB/N-NeuT. These are two models that are widely used mouse breast tumor models which more closely resemble human breast tumor development. The BALB-NeuT mouse strain contains rat mutant *Her2* and specifically expresses the constant active Her2 in the mammary glands by the MMTV promoter [119]. All of the 10 mammary glands of the mice will develop a breast tumor in 25 weeks [117]. The FVB/N-NeuT mouse strain contains Rat wild type *Her2* gene with the same promoter as BALB-NeuT [170]. This strain takes 20 to 40 weeks to develop 2.5 breast tumors on average. We used these two transgenic mouse tumor models to evaluate our vaccine candidates in a prophylactic setting.

Here, I will discuss our proof of concept experiments for the development of the prophylactic cancer vaccine based on the FS antigens. I mainly use one FS antigen from our list, SMC1A FS, which is shared by both human and mouse tumor cells.

2.2 Results

2.2.1Detection of SMC1A FS

2.2.1.1 Detection of SMC1A FS transcript

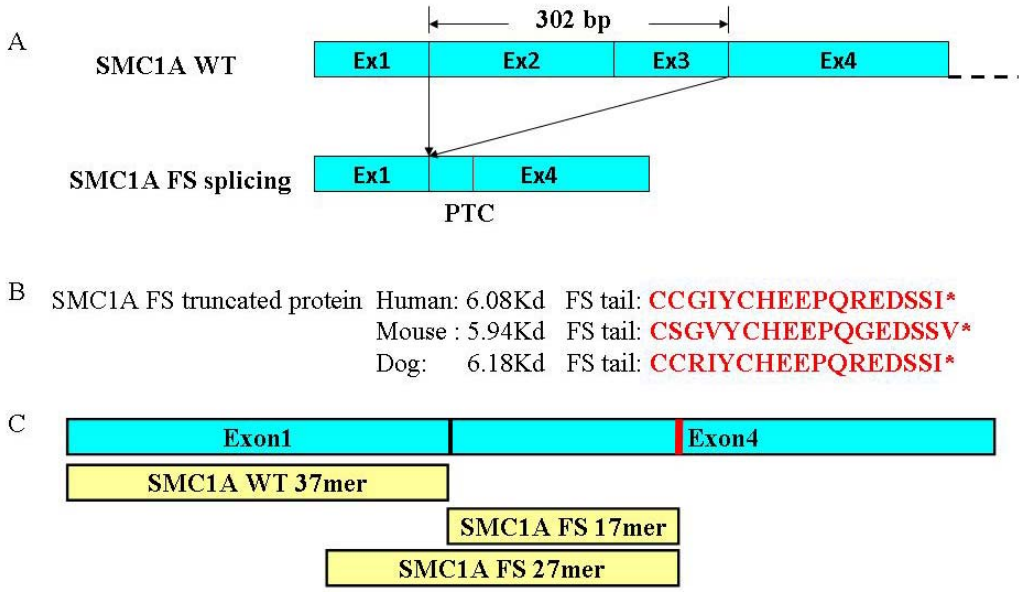

Figure2. 1 SMC1A WT and FS A. SMC1A WT and FS splicing transcript; B. FS peptide in human, mouse and dog; C. different SMC1A peptides used in following experiments. WT: wild type; FS: frame shift; PTC: premature termination codon

Table 2. 1 Alignment of mouse and human SMC1A exon 4. A. DNA sequence alignment of mouse and human SMC1A exon 4. There is 90% identity. B. Alignment of peptide sequence of third reading frame (FS) of SMC1A exon 4. Red bars present the stop codons. There is 74% identity of the entire sequence and 76% identity of the FS peptides. C. Alignment of peptide sequence of first reading frame (WT) of SMC1A exon 4. There is 100% identity.

A

```
mouse SMC1A exon4  1    GGTGCAGTGGAGTCTATTGCCATGAAGAACCCCAAGGAGAGGACAGCTCTGTTTGAAGAA  60
                        ||||| ||||| ||||||||||||||||||||||| ||||||||||||||| ||||||||
human SMC1A exon4  1    GGTGCTGTGGAATCTATTGCCATGAAGAACCCCAAAGAGAGGACAGCTCTATTTGAAGAG  60

mouse SMC1A exon4  61   ATTAGTCGCTCTGGGGAACTTGCACAGGAGTATGACAAGCGAAAGAAAGAAATGGTGAAG  120
                        |||||||| |||||||| || || ||||||||||||||||||||||| ||||||||||||
human SMC1A exon4  61   ATTAGTCGTTCTGGGGAGCTGGCGCAGGAGTATGACAAGCGAAAGAAGGAAATGGTGAAG  120

mouse SMC1A exon4  121  GCTGAAGAAGACACACAGTTTAATTATCATCGCAAGAAAAATATTGCAGCTGAACGAAAG  180
                        |||||||| ||||||||||||||||| |||||||||||||||||||| |||||||| |||
human SMC1A exon4  121  GCTGAAGAGGACACACAGTTTAATTACCATCGCAAGAAAAATATTGCGGCTGAACGCAAG  180

mouse SMC1A exon4  181  GAGGCCAAACAAGAAAAGGAAGA  203
                        || || || || || || |||||
human SMC1A exon4  181  GAAGCAAAGCAGGAGAAAGAAGA  203
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B

```
mouse SMC1A exon4  1   CSGVYCHEEPQGEDSSVRNSLWGICTGVQAKERNGEGRRHTVLSSQEKYCSIKG  54
THIRD reading frame    C G+YCHEEPQ EDSS+R+S WG   GVQAKE NGEGR HTVL SQEKYC I+G
human SMC1A exon4  1   CCGIYCHEEPQREDSSIRDSFWGAGAGVQAKEGNGEGRGHTVLPSQEKYCGIQG  54
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C

```
mouse SMC1A exon4  1   GAVESIAMKNPKERTALFEEISRSGELAQEYDKRKKEMVKAEEDTQFNYHRKKNIAAERK  60
FIRST reading frame    GAVESIAMKNPKERTALFEEISRSGELAQEYDKRKKEMVKAEEDTQFNYHRKKNIAAERK
human SMC1A exon4  1   GAVESIAMKNPKERTALFEEISRSGELAQEYDKRKKEMVKAEEDTQFNYHRKKNIAAERK  60

                   61  EAKQEKEE  68
                       EAKQEKEE
                   61  EAKQEKEE  68
```

The wild type SMC1A has multiple important functions in stabilizing sister chromatids during DNA replication, DNA repair in the ATM pathway and controlling gene expression. The SMC1A FS transcript was first detected by bioinformatics analysis by Bu and Johnston. The SMC1A FS transcript was present in 3 different human tumor EST libraries: mammary gland, skin and eye tumors. The SMC1A FS transcript is generated by directly splicing exon1 to exon4 of the SMC1A gene. The FS transcript is 302 base pairs shorter than the WT by missing exon2 and exon3 (Figure2.1). The abnormal splice changes the reading frame of exon4 and generates a PTC. The FS transcript encodes a truncated 54 amino acids protein including 37 amino acids of the wild

type sequence and 17 amino acids of the FS peptide tail at C-terminus. The predicted molecular weight of the truncated protein is 6.08 KD. The SMC1A is a highly conserved gene. Alignment of both DNA sequence and peptide sequence of the WT exon4 between mouse and human SMC1A showed the 90% and 100% identity respectively. It is interesting that the third reading frame of the exon 4 of mouse and human SMC1A also showed 74% identity (Table 1.1). The molecular weight of the FS truncated proteins in mouse is 5.94KD. To validate the bioinformatics analysis in tumor cDNAs, I designed a pair of flanking primers located on exon1 and exon4. The pair of primers can amplify both WT and FS transcripts in the same RT-PCR reaction with a 302bp difference from missing exon2 and exon3 in the FS transcript. We detected SMC1A FS in all human tumor cDNAs by endpoint RT-PCR with the flanking primers. These results came from a variety of cDNAs including 8 commercially primary breast tumor cDNAs (Figure 2.2 A) and 33 breast tumor cell lines (Figure AP 2.1). We also detected a relative lower level of the SMC1A FS transcript in 2 commercially human normal mammary gland cDNAs (Figure 2.2 A) and a non-tumorigenic epithelial cell line MCF-10A and normal pancreatic epithelial cell line HPDE6 (Figure AP 2.1). To quantify the different FS transcript levels in tumor and normal tissues, we applied quantitative RT-PCR with a Taqman probe which specifically hybridized to the SMC1A FS transcript (the experiment was performed by Dr. Buendia, Jose Cano, CIM). This data was normalized by beta-actin RNA. Results demonstrated that the FS transcript level is frequently higher 2 to 72 fold in human primary breast

tumors compared to normal mammary gland tissues (Figure 2.2 B). To validate the bioinformatics prediction of the SMC1A FS transcript in mouse and dog, we also screened the SMC1A FS in mouse and dog primary tumors and normal tissues cDNAs (Figure 2.3 A, Figure 2.4), as well as different mouse tumor cell lines (Figure 2.3 B). Similar to the screen in human cDNAs, we detected the FS transcript in all tumor cDNAs, as well as in some normal tissues. The different RT-PCR results of B16F10 cell in Figure2.3 A and B were caused by the different PCR settings. In summary, the RT-PCR analysis of different tumors demonstrated the SMC1A FS transcript presents commonly in different tumors and the q-PCR analysis suggested that the SMC1A FS may be overexpressed in tumors.

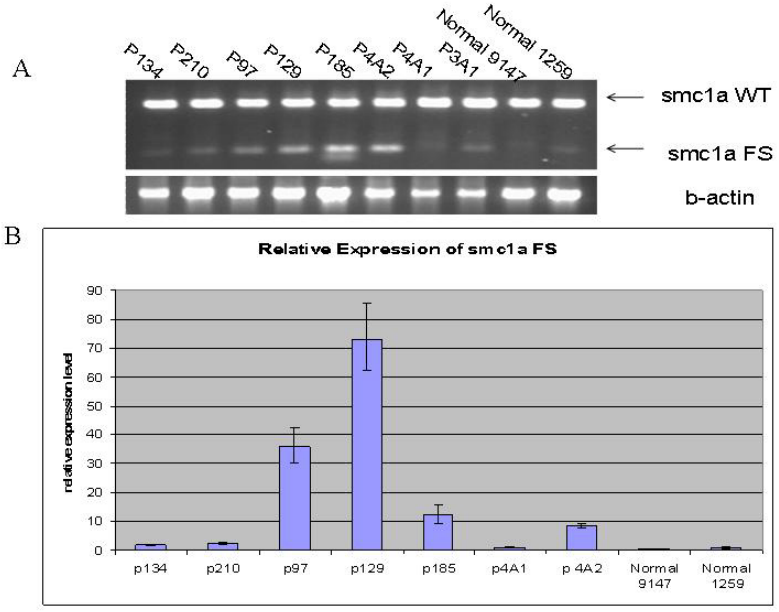

Figure2. 2 RT-PCR and Q-PCR analysis of SMC1A transcript in human primary brease tumors and normal mammary glands. A. RT-PCR analysis of 8 human primary breast tumor cDNA and 2 normal mammary gland cDNA; B. Q-PCR analysis of relative expression of SMC1A FS transcript in those cDNA samples. Expression level is normalized by beta actin. The experiment was performed by Dr. Buendia, Jose Cano, CIM.

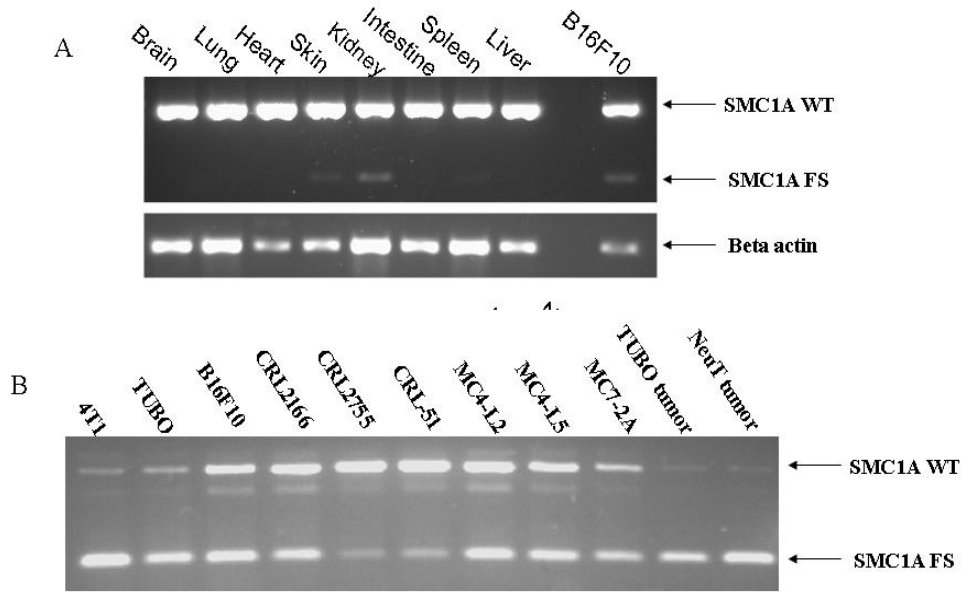

Figure2. 3 RT-PCR analysis of SMC1A FS transcript in mouse normal tissues and tumors. A. SMC1A FS transcript in C57BL6 mouse normal tissues and melanoma tumor cell line B16F10; B. SMC1A FS transcript in mouse tumor cell lines and mouse spontaneous tumor

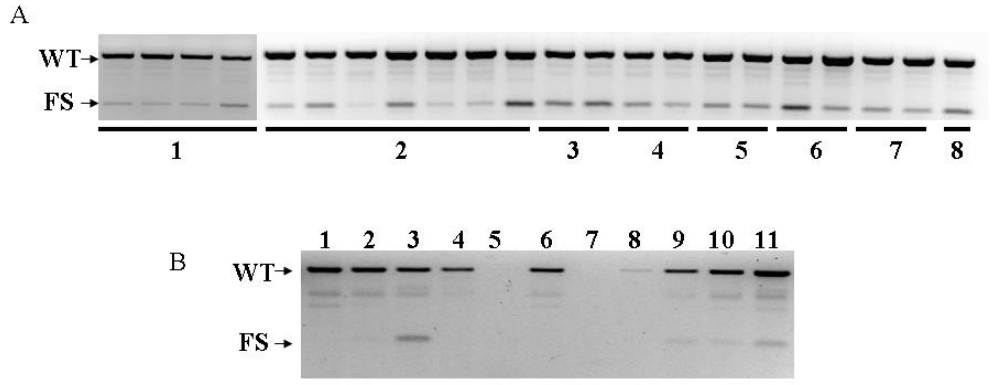

Figure2. 4 RT-PCR analysis of SMC1A FS transcript in dog primary tumors and normal tissues. A SMC1A FS in dog tumor. 1. melanoma; 2. osteosarcoma; 3.lymphosarcoma;4.hemangiosarcoma; 5. mammary; 6. mast cell tumor; 7. transitional cell carcinoma; 8. thyroid adenocarcinoma; B. SMC1A FS transcript in dog normal tissues. 1. cerebellum; 2. spleen; 3. mammary; 4. ovary; 5. pancreas; 6. thyroid; 7. small Intestine; 8. stomach; 9. tonsil; 10. heart; 11. liver

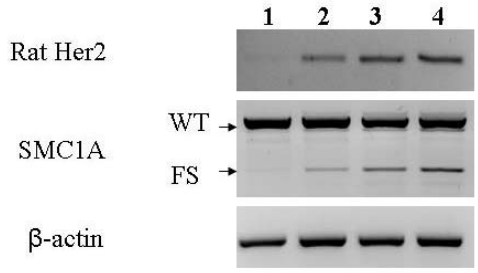

Figure2. 5 RT-PCR analysis of SMC1A FS transcript in normal mammary gland and breast tumors of FVB/N-NeuT . 1. normal adjacent mammary gland; 2. neoplasm of mammary gland; 3 and 4. established breast tumors

As described above, one important characteristic of the FS antigens for use in a prophylactic cancer vaccine is their expressions during early stages of tumor development. I analyzed the FS transcript of SMC1A in different stages of tumor in the FVB/N-NeuT mouse breast tumor model. cDNAs from a normal adjacent mammary gland, an early neoplasm and two fully developed breast tumors were analyzed (Figure2.5). As predicted, the expression of tumor derived transgene rat-her2 is increased along with tumor development. Interestingly, the SMC1 FS transcript level is also increased with tumor development. This indicates that the SMC1 FS transcript is trigged at an early stage of tumor development.

2.2.1.2 Analysis of SMC1A gene in Panc1 cell line

It is important to determine if there is a genetic mutation causing the FS transcript, because this will determine the level of the specificity of the SMC1A FS for the vaccine development. I did the investigations on the human Panc1 cell lines. Since the SMC1A FS transcript is a perfect splicing of the exon1 and exon4, I first checked if there is any genetic mutation at the splicing sites. I designed

primers to amplify a SMC1A gene fragment around the splicing sites of exon 2 and 3 in Panc1 tumor cell line. It starts from about 100 base pairs of 3' end of intron1 to 100 base pairs of 5' end of intron3 (Figure 2.6). Sequence results confirmed that there is no gene mutation in these areas. To investigate if there is exon2 and 3 deletions in the Panc1 cell line, I set up a long fragment PCR reaction to amplify the gene fragment from exon1 to exon4. However, there was no amplified band. This indicates that there is no big gene deletion including exon2 and exon3. This indicates the SMC1A FS transcript may be caused by the alternative splicing in Panc1 cells. The detail characterization need to be done to confirm that alternative splicing is the common causing of the SMC1A FS transcript in different tumors.

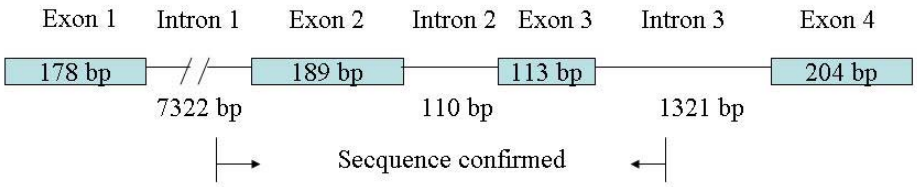

Figure2. 6 Analysis of SMC1A gene in Panc 1 tumor cell line. Sequence confirmed that there was no mutation around exon2 and exon3. There was no deletion from intron1 to intron3

2.2.1.3 Detection of the immune response to SMC1A FS peptide in tumor bearing mice

As described above, there are different levels to control the specificity of the FS antigens as the vaccine candidates. Some FS antigens may be caused by genetic mutations, such as translocations; some may be caused by

alternative splicing, such as SMC1A FS in our studies. The relevant specificity with regard to the vaccine development is whether this FS antigen is specifically exposed to the immune system by tumor cells. The way to determine this specificity is detecting endogenous immune response to the FS antigens. This analysis could not only demonstrate the specificity of the FS antigen, but also potential immunogenicity of the FS antigen, which is also important for the vaccine development. I analyzed both antibody reactivity and T cell responses to SMC1A FS peptide in the FVB/N NeuT mouse model. All FVB/N NeuT mice in the analysis were breeding females with end point tumor development according to our animal protocol. I detected antibody reactive to mouse SMC1A FS in sera from 5 of the tumor bearing females compared to 4 age matched no-tumor bearing breeding males which carried the same transgene (Figure 2.7). I also detected splenocytes specific to the SMC1A FS peptide in 3 of 4 tumor bearing females through an IFN-gamma ELISPOT assay (Figure 2.8). More importantly, there was no T cell activation of the FS portion (17aa) or the WT portion (37aa) alone. Only the combination of FS peptide and 10aa upstream of WT portion stimulated strong IFN-γ release splenocytes. I did not detect the IFN-γ releasing splenocytes in non-tumor bearing FVB/N male mice, nor in normal BALB/C and C57BL6 mice. This demonstrated that although some normal tissues also express the SMC1A FS, only the tumor cells expose the antigen to the immune system and induce the immune response.

The detection of the endogenous immune response to the SMC1A FS peptide only in tumor bearing mice demonstrated that the SMC1A FS antigen is specific to tumor cells at the immune response level. This also indicates there is an efficient T cell epitope in the junction of the WT and FS tail of the SMC1A FS truncated protein.

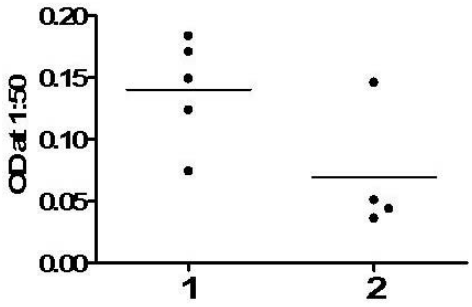

Figure2. 7 ELISA analysis of IgG activity to mouse SMC1A FS 27aa. 1. 26 weeks old tumor bearing FVB/N-NeuT females (n=5); 2. Age matched tumor free FVB/N-NeuT males (n=4). one-tail t-test $p<0.05$

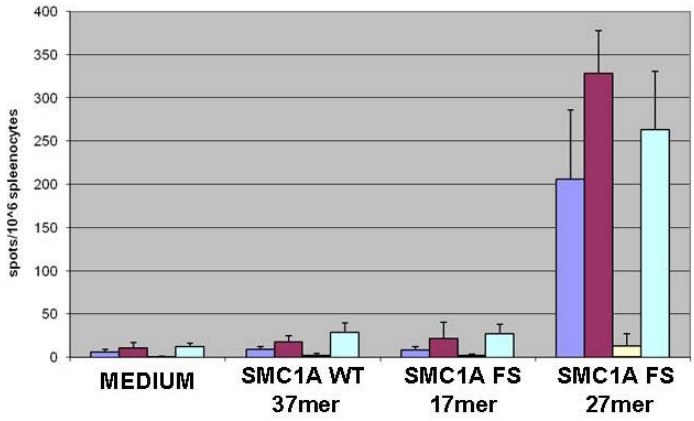

Figure2. 8 ELISPOT analysis of IFN-gamma release splenocytes. Splenocytes from four tumor bearing FVB/N-NeuT mice were restimulated with different peptides. The IFN-gamma releassed splenocyte were detected and counted with stand protocol

2.2.2 Protection of immunization of SMC1A FS in mouse tumor models

2.2.2.1 Epitope prediction

SMC1A FS is a very simple antigen. Mouse SMC1A FS peptide tail is a total of 17 amino acids long. I used Rankpep to predict the H2d, H2b and H2q binding epitopes. Those MHC I haplotypes belong to BALB/c, C57BL6 and FVB/N respectively, which were three mouse strains used for our mouse tumor models. Unlike most self protein antigens containing many epitopes, there is a handful epitopes of the SMC1A FS peptide, even by adding 10aa of WT peptide. Additionally, there is no epitope which has a predicted binding score higher than the binding threshold. The limited number of eptitopes and limited binding capacity make it a challenge to elicit efficient and appropriate immune responses against tumor. Besides the possible epitope prediction based on the binding capacity to different haplotypes, there are only two predicted epitopes based on proteasome cleavage prediction and both of them are located at the junction of WT and FS peptide. These results correspond to the detected T cell immune response in tumor bearing mice.

The SMC1A FS epitope predication in human population presents a similar situation. With the IEDB analysis, there are only 4 epitopes that could be recognized by four rare HLA types. One of them is located at the junction of WT and FS peptide. However, based on the proteasome cleavage prediction, there are four epitopes, 2 of them are located at the junction of WT and FS peptide, and another two are located at FS tail.

2.2.2.2 Optimization of Immunization

I mainly used genetic immunization with gene gun in most of the in vivo protection analysis. Later on, I also boosted the immune response with peptides or recombinant proteins. It is widely accepted that with the appropriate regimen and adjuvant, genetic immunization can efficiently elicit Th1 immune response [143, 145]. The combination of genetic immunization and protein boost is also the most efficient immunization regimen. Additionally, genetic immunization with the gene gun is an efficient method to screen our FS antigen candidates in the future.

The efficiency of an immunization regimen varies among different strains of mice with different genetic backgrounds relative to their immune system. I needed to adjust the immunization regimen in different models. Predicting the level of protection based on the immune response alone is also difficult. Therefore, I usually directly evaluated the protection of different regimens in mouse tumor models to optimize the immune regimen. This is a more direct method to optimize the efficiency of the immunization regimen.

Setting up the correct control is also important for optimization. In the study, there was a negative control for tumor inhibition to evaluate the protection of FS antigen and a positive control of immune response to evaluate the efficiency of immunization. I tried different controls over the course of the study. Human alpha-1 antitrypsin (AAT) is the first control antigen I tried. This is a high immunogenicity antigen and is irrelevant for tumor inhibition. The high immunogenicity of AAT makes it easy to elicit a strong immune response.

Consequently, it is hard to evaluate the efficient of different immunization regimens. I also tried another control antigen, CPV172. This is a protein from cowpox virus studied in our lab [171]. CPV172 can efficiently induce a T cell immune response. I used this antigen to optimize the regimen for eliciting a T cell immune response to SMC1 FS. Later I also used empty vectors and no-antigen fused recombinant protein for negative controls.

The adjuvant was another important factor for both protein and genetic immunizations. The choice of adjuvant is expanded with the development of genetic immunization with the gene gun [143-145]. The conventional micro gold delivery method can only deliver 1ug DNA molecules, such as plasmids. I tried two adjuvants for genetic immunization: a plasmid expressing mouse Granulocyte-macrophage colony-stimulating factor (GM-CFS) and two plasmids expressing heat-labile enterotoxins (LT). GM-CSF is a hematopoietic cytokine. It is widely use as an adjuvant in cancer vaccine trials in both humans and mice. Local administration of GM-CSF enhances the recruitment of dendritic cells and also induces their maturation [172]. This cytokine also has a function to activate macrophages, granulocytes and NKT cells. LT is another strong adjuvant that can elicit a Th1 immune response with the genetic immunization [173]. After we developed charged gold and nano gold delivery methods [146], we could efficiently deliver small nucleotides for genetic immunization, such as CpG oligodeoxynucleotides (CpG ODNs). CpG is another widely used adjuvant in both infectious disease and cancer vaccines. This adjuvant enhances the immune

response by triggering Toll-like receptor 9 in antigen presenting cells and inducing the releasing of Th1 cytokines. Both CpG 2395 and CpG 2216 were tested. CpG2216 belongs to CpG class A, which can active NK cells and induce IFN-alpha production in plasmacytoid dendritic cell precursors; CpG2395 belongs to CpG class C and it can active B cells and NK cells, as well as induce IFN-alpha production of human peripheral blood mononuclear cells. Both CpGs are directly compared in mouse tumor model. I also tried a novel adjuvant for genetic immunization: B2L, the virus protein identified in *parapoxvirus ovis[162]*. Besides its anti-tumor activity mentioned earlier, the adjuvant function of B2L mainly relies on the attraction of antigen presenting cells (mainly DC). B2L can significantly increase the immune response when it is co-immunized with antigens [162].

2.2.2.2.1 Dosage optimization

There are many factors that need to be optimized in order to elicit an appropriate immune response. The dosage of an antigen is one of the most important factors in the immunization. Especially in the primary immunization, different dosages of antigen will activate different proportions of high affinity T cells and low affinity T cells. Consequently, the dosage will affect the efficiency of an anti-tumor immune response. Instead of setting up a complex immune response analysis, such as titration ELISPOT, to evaluate the proportion T cells with varying affinity that have been activated by different dosages of primary immunization; I designed a quick immunization regimen with one

primary genetic immunization following tumor challenge. This strategy can directly reveal the best primary immunization dosage that necessary to elicit an efficient anti-tumor immune response by FS genetic immunization. In the dosage optimization experiments, I used the combination of two FS antigens with known protection: 1-78 and 6-21. 1-78 and 6-21 are FS antigens found in different human and mouse tumor cells, and they provide protection in different mouse tumor models. Besides the non-treated group and the AAT negative control group, there were four groups immunized with 1.5ug, 0.1ug, 20ng and 5ng of combined FS antigens. After 5 days of one genetic immunization with different dosages, all of the mice were challenged with 10^6 B16F10 tumor cells subcutaneously. Interestingly, the tumor growth curve revealed that the group immunized with 20ng of FS antigens received the best protection. All of the other groups did not show protection by primary immunization (Figure 2.9). High amount of antigen apparently are not protective. Therefore the 20ng of primary genetic immunization was chosen as the optimized dosage for the following experiments. I also compared the genetic immunization alone and genetic immunization with protein boost (Figure 2.10). It revealed that the protein boost did not improve the protection with a pool of FS antigens of 1-78 and 6-21.

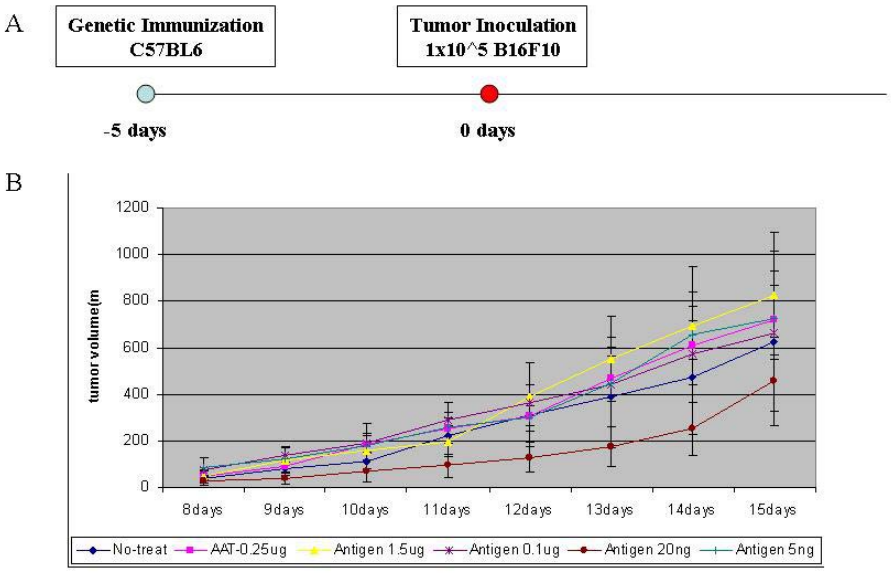

Figure2. 9 Dosage optimization of FS antigen. A. Immunization regimen. B. tumor growth curve of different groups as labeled. 5 mice per group; antigen group were immunized with different dosage of pooled 6-21 and 1-78 as indicated; GMCSF as adjuvant for genetic immunization; error bar are standard error.

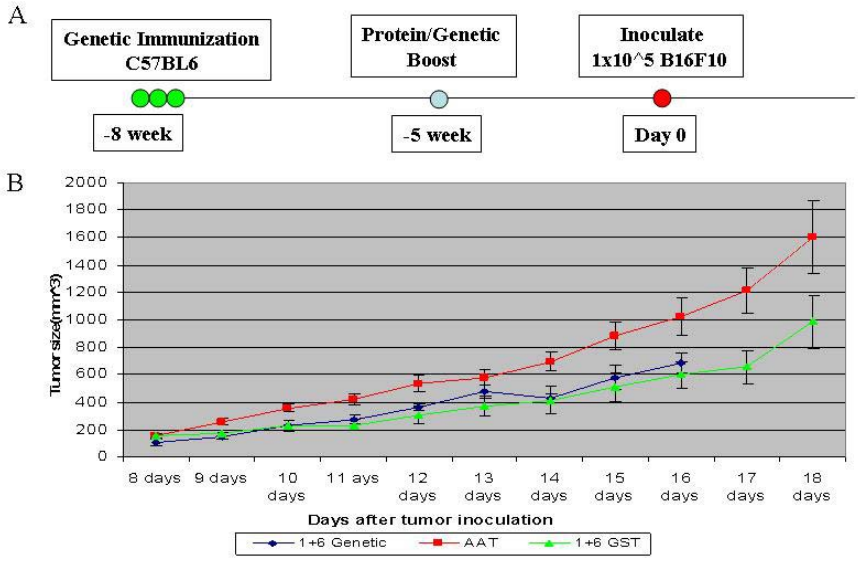

Figure2. 10 Comparing different immunization regimens. A. immunization regimen. B. tumor growth curve of different groups. 5 mice per group; AAT group as negative control; vaccine group were genetic immunized pool of 20ng each of 1-78 and 6-21; protein boost were a pool of 5ug each recombinant proteins of GST fused 1-78 FS peptide and GST fused 6-21 FS peptide; GMCSF as genetic adjuvant and IFA as protein adjuvant; error bar are standard error.

2.2.2.2.2Antigen optimization

Both endogenous immune responses and epitope prediction showed that the potential protective epitope was located at the junction of the WT and FS peptide. To confirm the prediction and decide which form of FS antigen to further study, I compared the protection of SMC1A FS peptide (17aa) and SMC1A WT+FS peptide (10aa WT with 17aa FS) in the B16F10/C57BL6 mouse tumor model. With the prophylactic immunization setting, the tumor growth curve of the SMC1A FS immunization group is no different from the negative control group, while the SMC1A WT+FS group exhibited the inhibition of tumor growth (Figure 2.11). These results confirmed the prediction that the immunogenic epitope was

located at the fusion of the WT and FS peptides. I decided to use SMC1A WT+FS as a vaccine candidate for further investigation. **In following description, I will use SMC1A FS to represent the immunization of the 27amino acid SMC1A WT+FS peptide for convenience.**

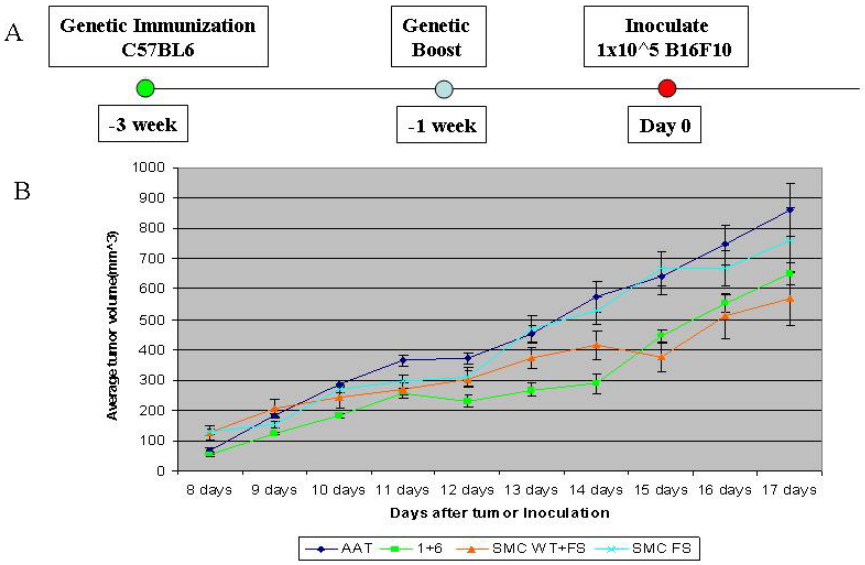

Figure2. 11 Optimization of SMC1A FS antigen. A. Immunization regimen. B. tumor growth curve of different groups as labeled. 5 mice per group; AAT were immunized with AAT as negative control; 1+6 were immunized with pool of 1-78 and 6-21; SMC FS were immunized with SMC1A FS 17mer peptide; SMC WT+FS were immunized with SMC1A FS peptide puls with 10 aa upstream WT peptide; GMCSF as genetic adjuvant; error bar are standard error

2.2.2.2.3 Adjuvant and regimen optimization

The most optimizations were developed in the B16F10/C57BL6 mouse model. With the genetic immunization of conventional micro gold particles, I compared adjuvant affects of LT and GM-CSF with the optimized dosage of SMC1A in the same setting (Figure 2.12). The different adjuvants did not affect tumor growth in the AAT control group. The LT adjuvant caused the loss of

protection in the 1-78 and 6-21 pooled group and it even promoted tumor growth in the SMC1A group. There was a higher IgG antibody reactivity to SMC1A FS in mice immunized with LT adjuvant (Figure 2.13). No antibody reactivity to 1-78 and 6-21 FS peptides was detected in both groups immunized with 1-78 and 6-21 FS peptides. There was no detectable IFN-gamma releasing splenocytes to any FS peptide in all groups in the ELISPOT assay. This indicated that antibody activity is not the important protective immune response in this model. I need to test adjuvants that can elicit more balanced immune responses.

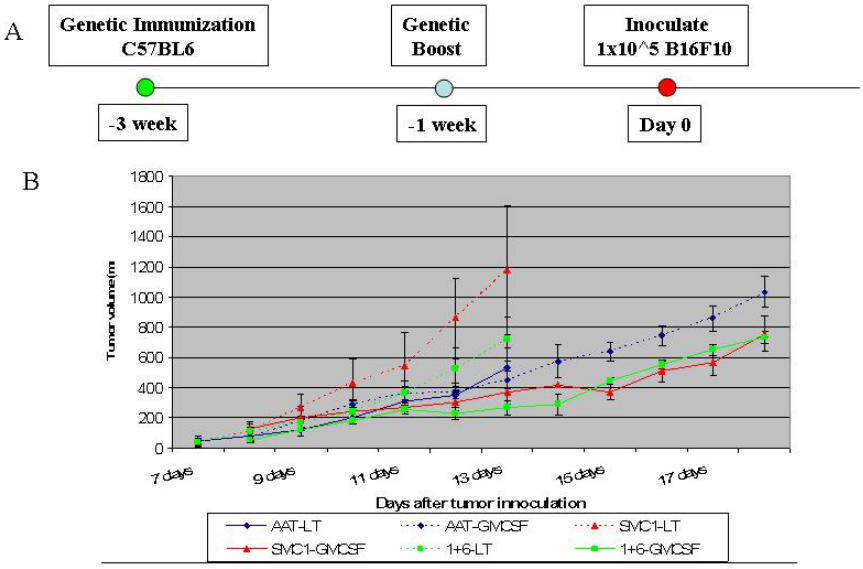

Figure2. 12 Optimization of genetic adjuvant. A. Immunization regimen. B. tumor growth curve of different groups as labeled. 5 mice per group; 20ng of each FS antigen or AAT per mouse; 1ug LT or GMCSF as adjuvant; AAT were immunized with AAT as negative control; 1+6 were immunized with pool of 1-78 and 6-21; SMC1 were immunized with SMC1A FS 27mer peptide; error bars are standard error

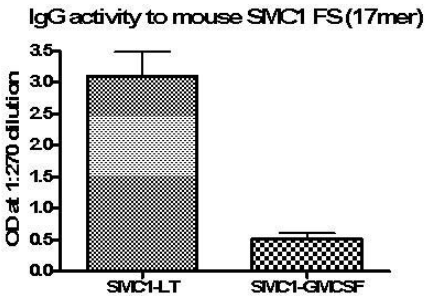

Figure2. 13 ELISA analysis of IgG activity against SMC1 FS 17mer peptide in mice immunized SMC1 FS with different adjuvants. 5 mice per group; duplicates of individual mouse endpoint serum with different group at 1:270 dilution; error bar are standard error of each group

A

*Total 6 groups and 5 mice per group.

*Pool antigens: 1-78, 6-21, cpv172 (all in UB+LS) and smc1 wt+FS(in UB and LCTT)

*Group setting: (dosage/mouse/immunization)

- Group1: 80ng pool antigens + 0.5ug GMCSF
- Group2: 80ng pool antigens + 0.5ug GMCSF + 5ug CpG 2395
- Group3: 80ng pool antigens + 0.5ug GMCSF + 5ug CpG 2216
- Group4: 80ng pool antigens + 0.5ug GMCSF + 0.5ug CpG 2216
- Group5: 80ng pool antigens + 5ug CpG 2216
- Group6: 80ng pool antigens + 0.5ug GMCSF + 5ug CpG 2216 (one immunization)

B

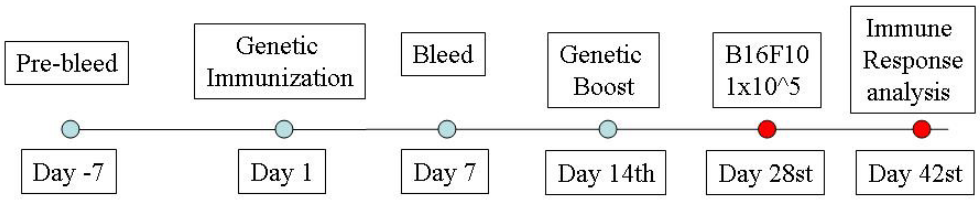

Figure2. 14 Experimental plan for immunization optimization. A. Group setting. B. immunization regimen

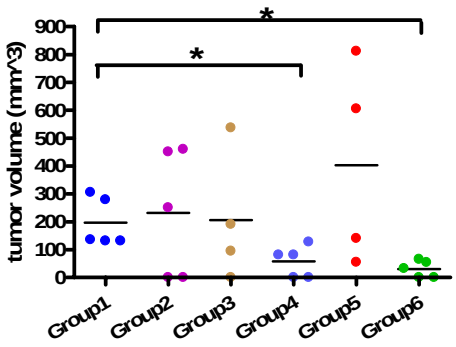

Figure2. 15 Individual tumor volume at 13 days after tumor inoculation. *: p<0.05

With the genetic immunization with charged microgold particles, I tested different combination of GMCSF and CpG as adjuvants followed by tumor challenge (Figure 2.14). To analyze the immune response, I ended the experiment before the tumor fully developed. By comparing the tumor growth in different groups to the immunization with GMCSF alone, only two groups exhibited the improvement of protection against tumor: the group4 with the primary and boost immunizations with combination of GMCSF and 0.5ug CpG2216 and the group6 that was just primary immunized with combination of GMCSF and 5ug CpG2216 (Figure 2.15). To investigate the immune response of the improved protection, I analyzed IgG isotype activity by ELISA and IFN-gamma releasing splenocytes by ELISPOT. The group that was primary and boost immunized with the combination of GMCSF and 0.5ug CpG2216 exhibited the balance of IgG subtype activity against SMC1A FS peptide by ELISA and T cell activity by ELISPOT, while the group that was just primary immunized with combination of GMCSF and 5ug CpG2216 exhibited no antibody activity but the high T cell activity (Figure2.16 and 2.17).

In summary, based on the efficacy of tumor inhibition, the immunization regimens of the group4 and group6 exhibited the most protection. It also suggested that both antibody and T cell immune response are important for the protection. For example, the group1 had strong antibody activity and low T cell activity, while the group5 had no antibody activity and high T cell activity. Neither of these groups showed as protection as the group4, which had more a balanced immune responses of antibody activity and T cell activity. It was interesting that the group6 showed the best protection while only exhibiting the strong T cell activity by one low dosage genetic immunization. In the infectious diseases vaccine development, the low dosage and long period resting time could elicit high avidity T cell response ([173] and communication with Dr. Stephen Johnston). This may be the same mechanism in the group6. Although there was no antibody response, the high avidity T cell response still could efficiently inhibit the tumor development. However, there was no immune response detected to the 6-21 and 1-78. And both group2 and group3 exhibited relative balanced immune responses, while there were no significant improvements of the protection. This indicates that the immune response analysis that I applied may be not sensitive enough to detect all of the anti-tumor activities. I need to improve the analysis in the future.

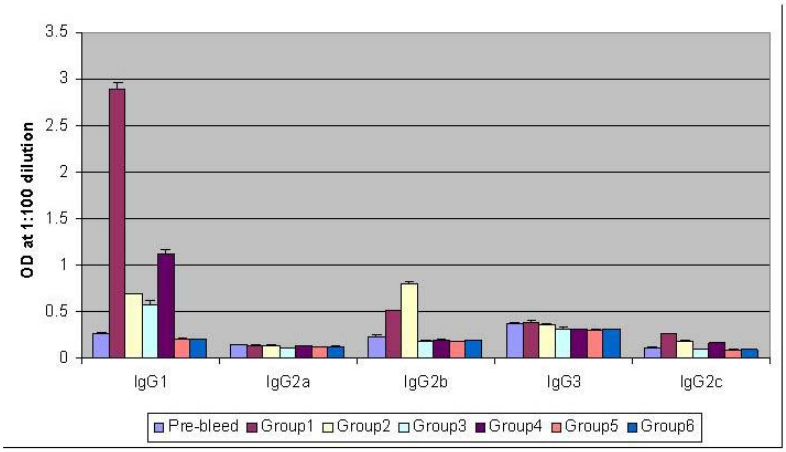

Figure2. 16 ELISA analysis of IgG subtype activity against SMC1A FS peptide of pooled endpoint serum of each group. Serum dilution 1:100; in duplicate

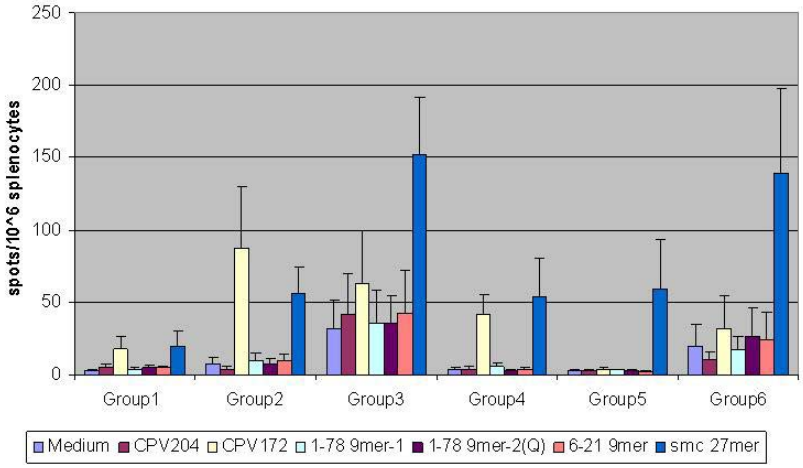

Figure2. 17 ELISPOT analysis of IFN-gamma released by splenocytes. Splenocytes from each mouse were incubated with different antigens in triplicate for 48 hours at 20ug/ml concentration and followed standard protocol for spots development; each column represents average spots of each group with different antigens; error bars represent standard error (SEM)

Later, I also compared the different immunization regimens of combination of SMC1A FS and CPV172 with different adjuvant compositions

with the protein boost (Figure 2.18). All groups demonstrated the robust IgG1 activity to SMC1A FS, while only the group with the adjuvant of CpG2216 and LTAB had the IgG2c (equivalent of IgG2a in C57BL6) activity (Figure 2.19). More detailed subtype analysis of the two groups with CpG plus GMCSF and CpG plus LTAB demonstrated that the latter adjuvant combination elicited a balanced IgG subtype activity with genetic immunization alone and an even more balanced activity with a protein boost (Figure 2.20). This indicated that the CD4 T help cells were successfully activated by the immunization regimen. There is no detectable SMC1A FS specific IFN-gamma releasing splenocytes in any of the groups.

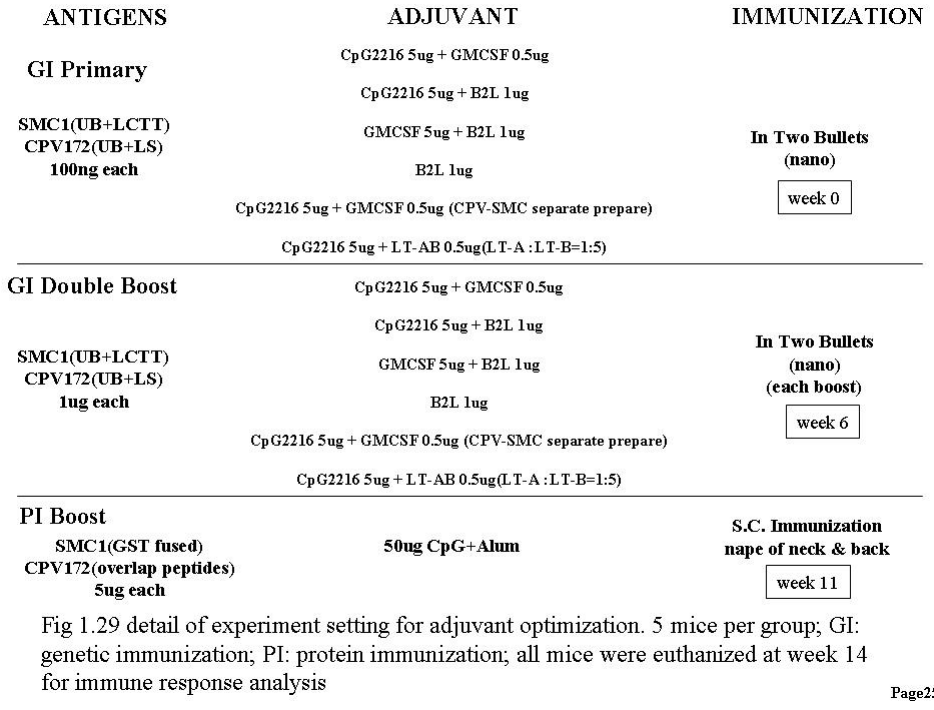

Fig 1.29 detail of experiment setting for adjuvant optimization. 5 mice per group; GI: genetic immunization; PI: protein immunization; all mice were euthanized at week 14 for immune response analysis

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Figure2. 18 Detail of experimental plan for adjuvant optimization. 5 mice per group; GI: genetic immunization; PI: protein immunization; all mice were euthanized at week 14 for immune response analysis

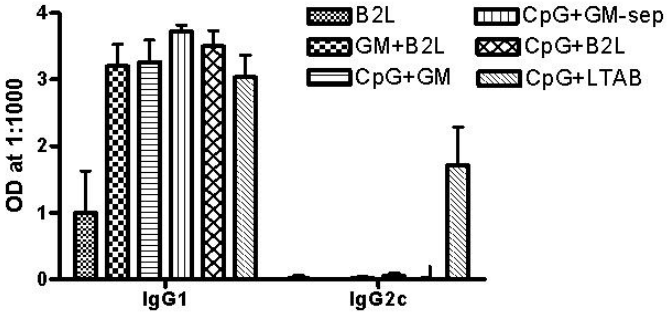


Figure2. 19 ELISA analysis of IgG subtype activity against SMC1A FS 27mer. Serum dilution 1:1000; each column represents average OD of each group with different IgG subtype as labeled; error bars are standard error

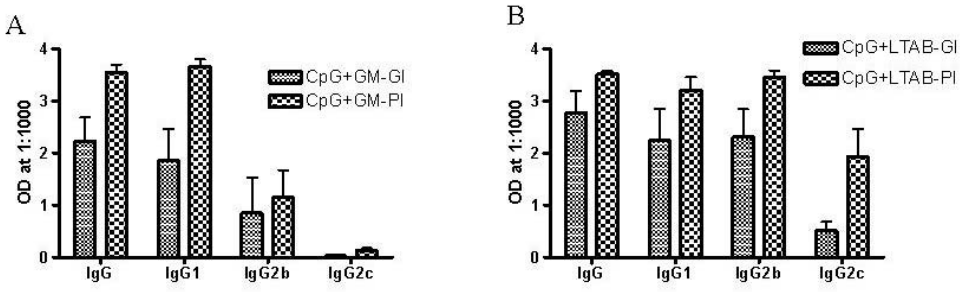


Figure2. 20 Detail subtype IgG analysis against SMC1A FS 27mer of two groups: A. subtype analysis of group use CpG and GMCSF as adjuvant; B. subtype analysis of group use CpG and LTAB as adjuvant. Each column represents average OD of each group with different IgG subtype as labeled; error bars are standard error

I also tried a regimen to knock down the Treg cells with cyclophosphamide treatment before the immunizations to enhance the cellular immune response. This approach failed to enhance the immune response or provide protection. The treatment needs to be optimized and more carefully administrated.

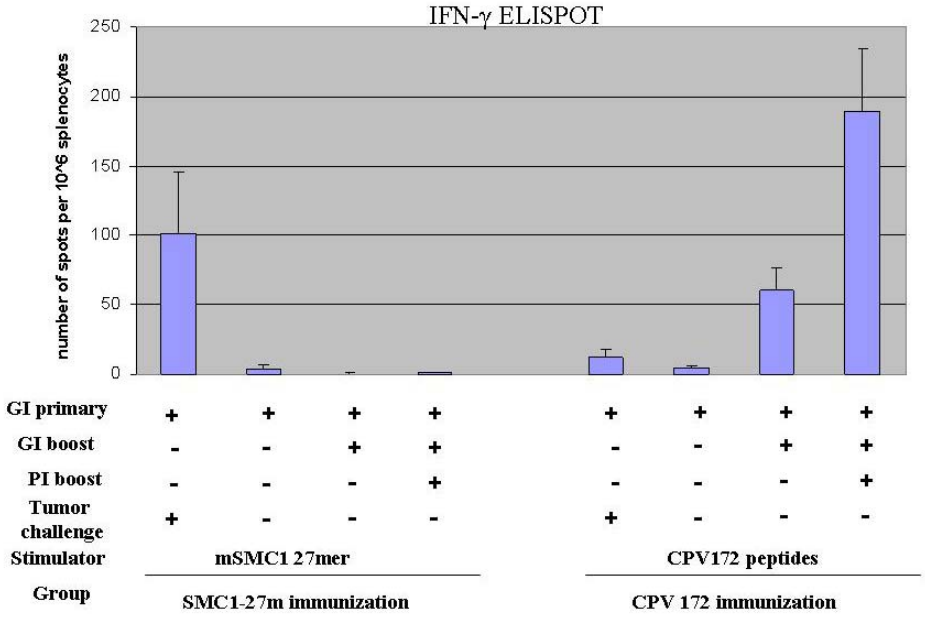


Figure2. 21 Summarize of IFN-gamma release splenocyte ELISPOT analysis of SMC1A FS and CPV172 immunization with different regimens

Summarizing all of the optimizations in C57BL6 mice, the results showed that the proper immunization regimens could robustly elicit specific IgG activity to SMC1A FS with a balanced IgG subtype, which indicates that the CD4 T cells were activated. On the contrary, there was no detectable cellular immune response measured by IFN-gamma ELISPOT of the splenocytes in all settings with immunization alone, while the cellular immune response to a positive control CPV172 was increased alone with an immunization intensity dependent increase as predicted (Figure2.21). However, the cellular memory immune response was successfully elicited by the primary genetic immunization with a low dosage of SMC1A FS and boosted more than 20 folds higher by the B16F10 tumor cell challenge (Figure 2.21).

In summary, the optimization suggested the following principles for FS antigens immunization:

1. There are possible protective epitopes located at the junction of the WT and FS peptide tails. Those also are specific tumor epitopes and it is helpful to include them into the FS immunization.

2. The humoral immune response could be robustly elicited by different immunization regimens.

3. Low primary immunization dosage is critical for eliciting the cellular immune response to the FS antigens. The antibody isotype switch indicates the stimulation of the $CD4^+$ T cell. The memory $CD8^+$ T cell immune response also could be elicited. However, there is no evidence of eliciting the active $CD8^+$ T cell by the immunization.

4. CpG2216 is the essential adjuvant for stimulating the efficient anti-tumor immune response. Both GMCSF and LTAB need to be combined with Cp2216 to maximize the adjuvant to enhance the anti-tumor immune response.

5. Protein based boosts did not show a positive effect to improve the anti-tumor immune response, and it did not have negative effect either.

Because C57BL6 is a TH1 immune response biased mouse, the detailed immunization regimens may still need to be further adjusted based in a more wild-type background and humans.

2.2.2.3 Evaluation of primary tumor prevention

During the optimization of the immunization, I have already demonstrated the protection provided by SMC1A FS, as well as other FS candidates. This work was primarily performed with the B16F10/C57BL6 mouse tumor model. To prove our concept of a broad prophylactic cancer vaccine, I also tested the protection in various mouse tumor models by immunization of SMC1A FS and other FS candidates. The initial optimization indicated that the general principles of the FS antigen immunization could be successful. The efficient immunization regimen is also dependent on the different genetic background of the immune system in different mouse models. The adjusting of the immunization was performed to evaluate the effect of tumor prevention in different mouse tumor models.

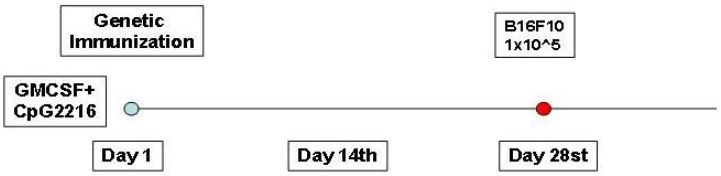
2.2.2.3.1 Protection in transplanted mouse tumor models

Both B16F10/C57BL6 and 4T1/BALB/c transplant mouse tumor models are the first models that were introduced in our studies. These models are useful to quickly evaluate our FS candidates.

2.2.2.3.1.1 Protection in B16F10/C57BL6 model

With the optimized immunization regimen (Figure 2.14), I evaluated individual FS candidates in this model (Figure 2.22). As predicted, all of the three FS candidates provided the protection by inhibiting the tumor development compared to CVP172 negative control group (Figure 2.23). Without the protein

boost, there was no detectable IgG activity as demonstrated in the previous optimization experiments (Figure 2.24).



Group Setting (10mice/group C57BL6) :

- Group1: CPV172 alone
- Group2: smc1wt-fs
- Group3: 1-78fs
- Group4: 6-21fs

- Dosage: (dosage/mouse) 20ng/antigen+0.5ug GMCSF+5ugCpG2216
- 4 shot/mouse
- Inoculation: 1X10^5 B16F10/mouse

Figure2. 22 Detail experimental plan for protection evaluation of FS antigens in B16F10/C57BL6 mouse melanoma tumor model

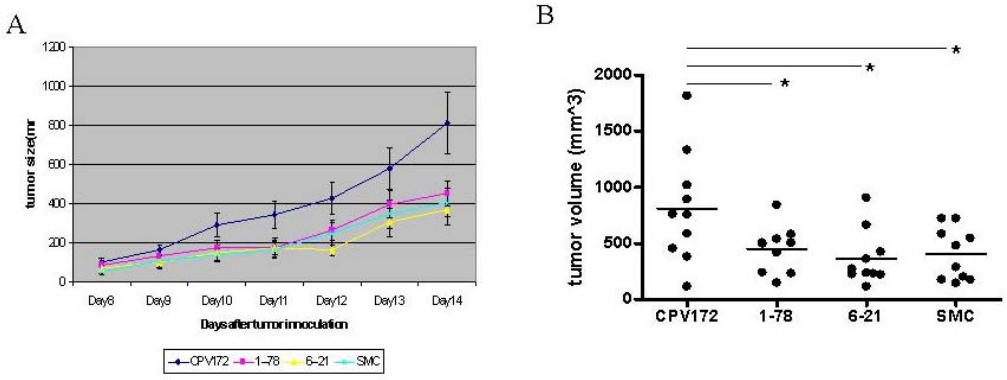
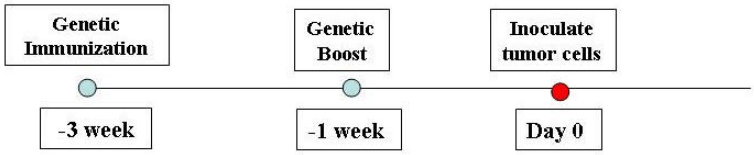


Figure2. 23 Tumor size measurement. A. Tumor growth curve. B. Individual tumor size of each mouse at 14 days after tumor inoculation. * p<0.05

2.2.2.3.1.2 Protection in 4T1/BALB/c model

4T1/BALB/c is another model in my study. I tested the protection with different immunization regimens along with the development of the

optimization. Only the 1-78 and SMC1A FS transcripts were detected in the 4T1 cells. In some experiments, I included the 6-21 for control. Before the dosage optimization, there was barely detectable protection of 1-78 in this model. The lower dosage immunizations significantly increased the protection (Figure 2.24 and Figure 2.25 A). Both SMC1 and 1-78 and 6-21 pooled groups exhibited the inhibition of tumor growth compared to the AAT negative control group. The specific IgG activity against SMC1A FS peptide was detected in the SMC1 group (Figure 2.25B); while there was no detectable IgG activity against 1-78 and 6-21 peptides in the 1-78 and 6-21 pooled group.



Group Setting (10mice/group BALB/c) :

- Group1: AAT
- Group2: smc1wt-fs
- Group3: 1-78fs +6-21fs

- Dosage: (dosage/mouse) 20ng/each antigen+1ug GMCSF
- 4 shot/mouse
- Inoculation: 5X10^3 4T1/mouse

Figure2. 24 Detail experimental plan for protection evaluation of FS antigens in 4T1/BALB/c mouse breast tumor model

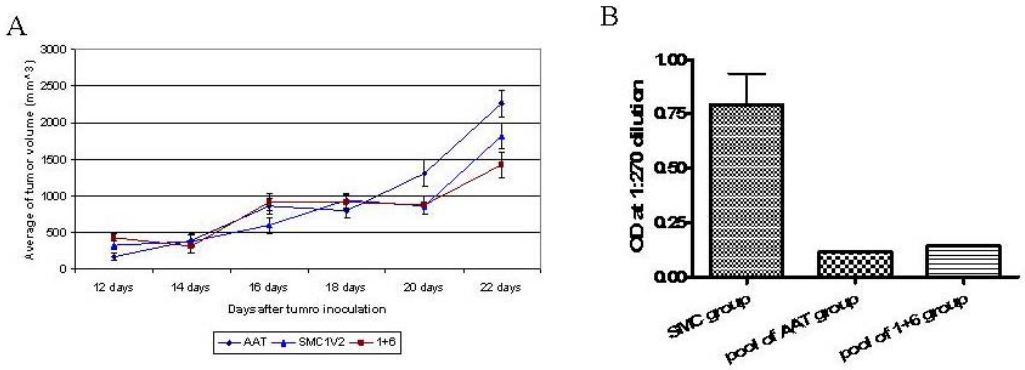


Figure2. 25 Protection analysis of 4T1/BALB/c mouse breast tumor model. A. Tumor growth curve. B. ELISA analysis of IgG activity against SMC1A FS 27mer

I also tested the regimen of one genetic immunization alone with GMCSF and CpG2216 in the 4T1/BALB/c model (Figure 2.26). This experiment was designed for evaluating the prevention of the tumor in BALB-NeuT breast tumor model. Those transgenic mice failed to develop spontaneous breast tumors after they were 30 weeks old for unknown reasons. As an alternative, I used these mice as a transplanted tumor model by challenging these mice with 4T1 cells to evaluate the long term prevention of our prophylactic cancer vaccine. As designed, all mice were immunized with different antigens at 4 to 5 weeks old and were challenged with $5x10^3$ 4T1 cells at 26 to 38 weeks old. One primary immunization can efficiently inhibit tumor development after more than 3 months as seen on the tumor growth curve (Figure 2.27). There was no detectable IFN-gamma released splenocytes to any of the FS peptides and there was low IgG activity to SMC1A FS 27mer in the SMC1A FS immunized group.

Group Setting (BALB/c) :

- Group1: CPV172 12 mice 26-31weeks old
- Group2: SMC1A FS 4 mice 26 weeks old
- Group3: 1-78fs +6-21fs+SMC1A FS+CPV172 12 mice 32-38 weeks old

- Dosage: (dosage/mouse)
  20ng/each antigen+0.5ug GMCSF+5ug CpG 2216
- 4 shot/mouse
- Inoculation: 5X10^3 4T1/mouse

Figure2. 26 Detail experimental plan for protection evaluation of FS antigens in 4T1/BALB/c mouse breast tumor model

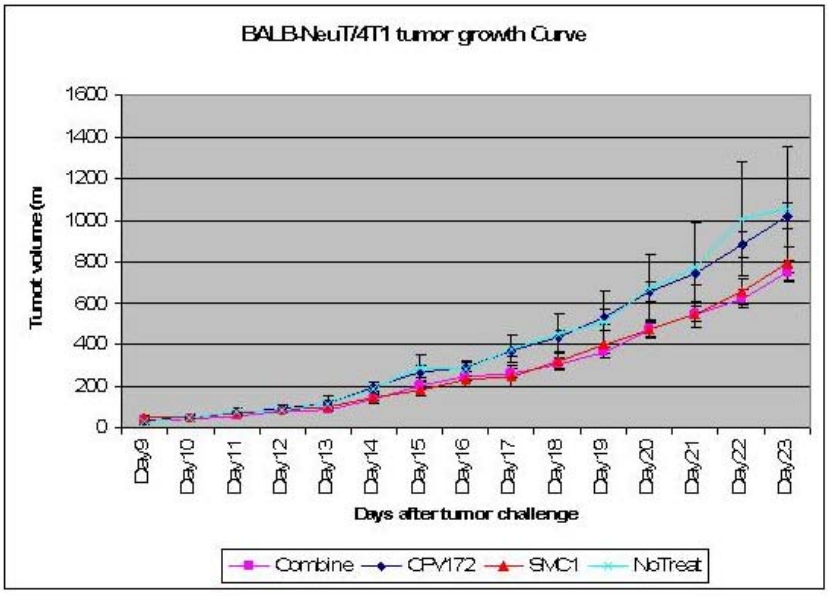


Figure2. 27 Tumor growth curve of each group. Error bars are standard error

In summary, I demonstrated the prophylactic protection of SMC1A FS antigen in two different mouse transplant tumor models, as well as other FS antigens. The mouse transplant tumor models are useful for quick functional

vaccine candidate screening. However, since the tumor development in these models is so aggressive, they are not suitable to test the concept of the prophylactic cancer vaccine. Therefore, we tested the prophylactic cancer vaccine in transgenic mouse tumor models.

2.2.2.3.2 Protection in transgenic mouse tumor models

The transgenic mouse tumor models are widely used in different types of cancer research. The mice in these tumor models spontaneously develop different types of tumors due to different gene modifications. These mouse tumor models more closely mimic the natural tumor development compared with the traditional tumor cell transplant mouse tumor models. The transgenic models offer an alternative to the transplanted mouse models to test the prophylactic cancer vaccines. However they are still limited relative to tumor development in humans in that many cells over a short period are converted to pre-tumor cells.

2.2.2.3.2.1 Protection in the FVB/N NeuT model

All mice were genetically immunized with different antigen components and GMCSF and CpG at 4 to 5 weeks old. The tumor development was monitored weekly (Figure 2.28). I did not detect 6-21 FS transcript in the FVB/N NeuT tumors as was the case for 4T1 cell line. Both CPV172 and 6-21 immunized groups were negative control groups. To evaluate the protection, I analyzed the tumor free rate and tumor multiplicity curve along with the tumor development in different groups (Figure 2.29 and Figure 2.30). Over all, both 1-78 and SMC1 FS

antigens exhibited inhibition of breast tumor development in the FVB/N-NeuT mouse model compared to the no-treatment group. However, with all individual antigen immunization, there was no difference in the tumor inhibition among these four groups. The negative control groups CPV172 also exhibited the significant tumor inhibition compared to the no-treatment group. This may be caused by the adjuvant of CpG and GM-CSF treatment, which can enhance the innate immune response. I will describe this effect in another chapter. Therefore, the adjuvant effect masked the protection of the SMC1A FS and 1-78 compared to the other two negative control groups. It is interesting that the pool of them with other negative antigens (CPV172 and 6-21) significantly improved the tumor inhibition compared to the 6-21 group. This implies that the pool of the SMC1A FS and 1-78 may have the additive protection. This need to be further investigated.

Group Setting (FVB/N-NeuT) :

- Group1: No-treated 5 mice
- Group2: CPV172 14 mice
- Group3: 6-21fs 11 mice
- Group4: 1-78fs 13 mice
- Group5: SMC1A FS 17 mice
- Group6: 1-78fs +6-21fs+SMC1A FS+CPV172 17 mice

- Dosage: (dosage/mouse)
  20ng/each antigen+0.5ug GMCSF+5ug CpG 2216
- 4 shot/mouse

Figure2. 28 Detail experimental plan for protection evaluation of FS antigens in FVB/N-NeuT mouse breast tumor model.

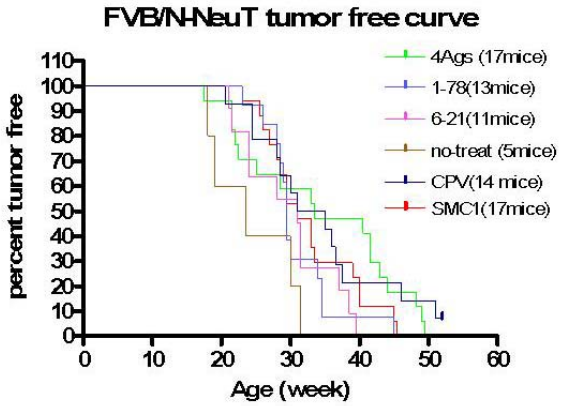


Figure2. 29 Tumor free curve of different group in FVB/N-NeuT model. No-treated group vs. SMC1 group, 4 antigens group and CPV172 group p<0.05

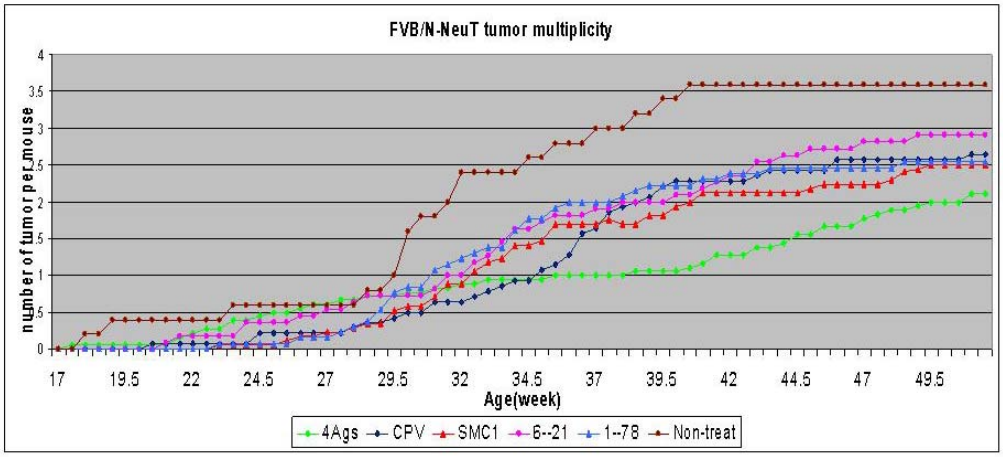
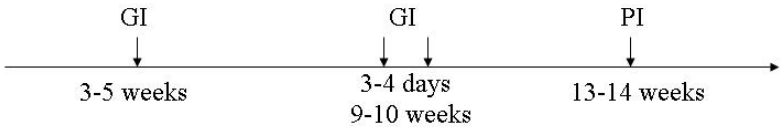


Figure2. 30 Tumor multiplicity curve of different groups of FVB/N-NeuT model.

2.2.2.3.2.2 Protection in BALB NeuT model

The BALB-NeuT mouse carries the active form of rat Her2, which starts to be over-expressed in mammary gland at 4 weeks old. The BALB-NeuT mouse starts to develop the first palpable tumor around 11 weeks old. Because of

the different genetic background of the immune system in BALB/c mice and different tumor development period, I tested different immunization regimens in the BALB-NeuT mouse tumor model to achieve the best protection (Figure 2.31). This revealed that the regimen of a genetic primary immunization followed by two genetic boosts and one protein based boost was the best immunization regimen for inhibition of tumor development in this model (Figure1.43). The combination of LTAB and CpG was the adjuvant for the genetic immunization and CpG plus Alum was the adjuvant for the protein based immunization.



Group1 7 mice	20ng SMC1A 5ugCpG+0.5ugGMCSF		
Group2 8 mice	100ng SMC1A 5ugCpG+0.5ugGMCSF	1ug SMC1A 5ugCpG+0.5ugGMCSF	5ug GST-SMC1A 50ugCpG+Alum
Group3 11 mice	100ng SMC1A 5ugCpG+0.5ugLTAB	1ug SMC1A 5ugCpG+0.5ugLTAB	5ug GST-SMC1A 50ugCpG+Alum
Group4 8 mice	1ug SMC1A 5ugCpG+0.5ugLTAB	1ug SMC1A 5ugCpG+0.5ugLTAB	5ug GST-SMC1A 50ugCpG+Alum

Figure2. 31 Detail experimental plan for immunization regimen optimization of SMC1A FS in BALB-NeuT mouse breast tumor model

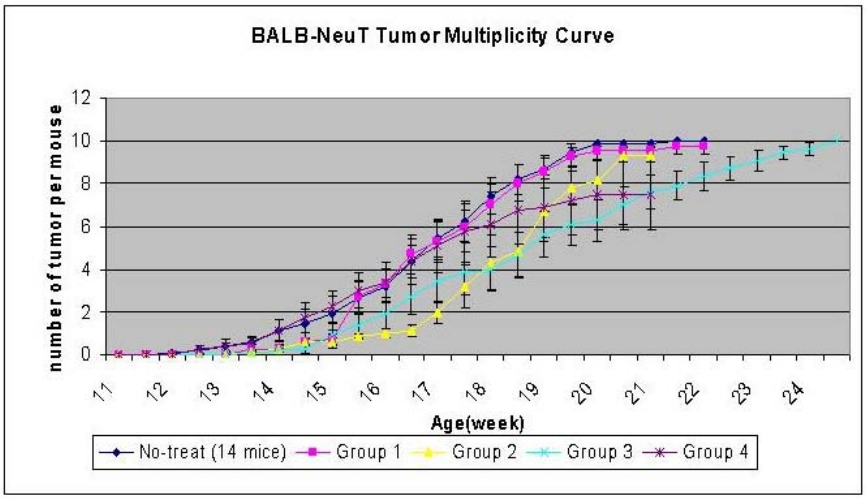


Figure2. 32 Tumor multiplicity curve of different groups with different immunization regimens.

I also tried different adjuvant combinations with CpG and B2L for the genetic immunization (Figure2.33). B2L was found to inhibit of tumor growth in the transplant mouse tumor model B16F10/C57BL6. Because of the anti-tumor activity of the B2L, the negative control group also had the partial protection (Figure 2.34).

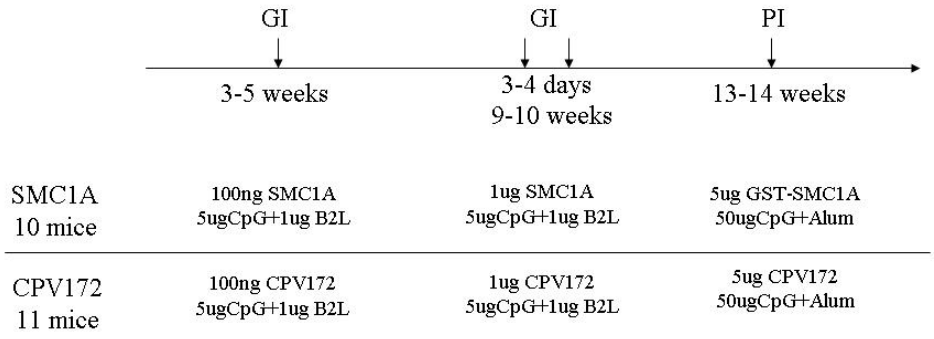


Figure2. 33 Detail experimental plan for immunization regimen optimization of SMC1A FS in BALB-NeuT mouse breast tumor model with CpG plus B2L as adjuvant

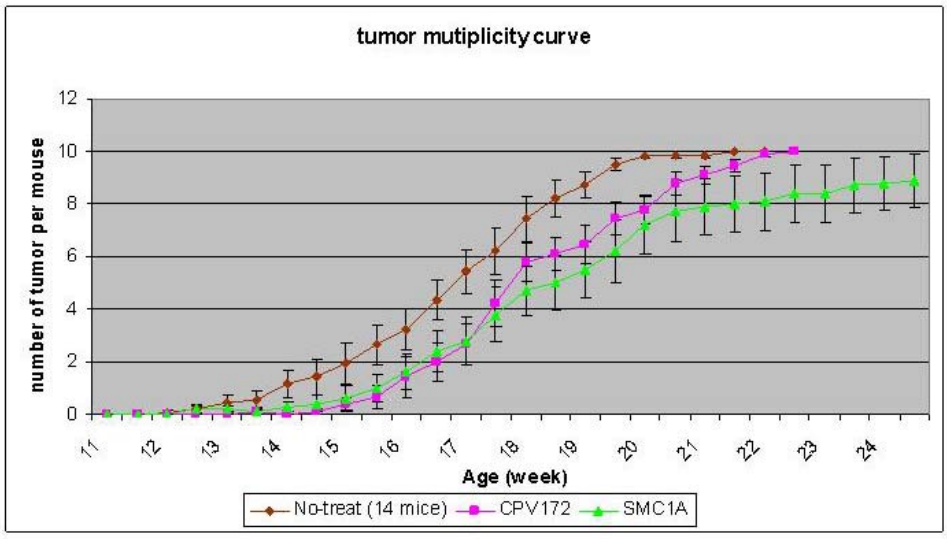


Figure2. 34 Tumor multiplicity curve of SMC1A group and CPV172 group with CpG plus B2L as adjuvant

With the optimized immunization regimen (Figure 2.35), the prophylactic immunization of SMC1A FS exhibited not only significant inhibition of first tumor initiation (Figure 2.36 A), but also the significant inhibition of tumor progression of developing 10 independent breast tumors (Figure 2.36 B). The tumor multiplicity curve also exhibited the significant protection by the prophylactic immunization of the SMC1A FS (Figure 2.37).

	GI 3-5 weeks	GI 3-4 days 9-10 weeks	PI 13-14 weeks
SMC1A 18 mice	100ng SMC1A 5ugCpG+0.5ugLTAB	1ug SMC1A 5ugCpG+0.5ugLTAB	5ug GST-SMC1A 50ugCpG+Alum
Negative 17 mice	100ng empty plasmid 5ugCpG+0.5ugLTAB	1ug empty plasmid 5ugCpG+0.5ugLTAB	5ug GST 50ugCpG+Alum
No-treated 14 mice			

Figure2. 35 Detail experimental plan for protection evaluation of SMC1A FS in BALB-NeuT mouse breast tumor model

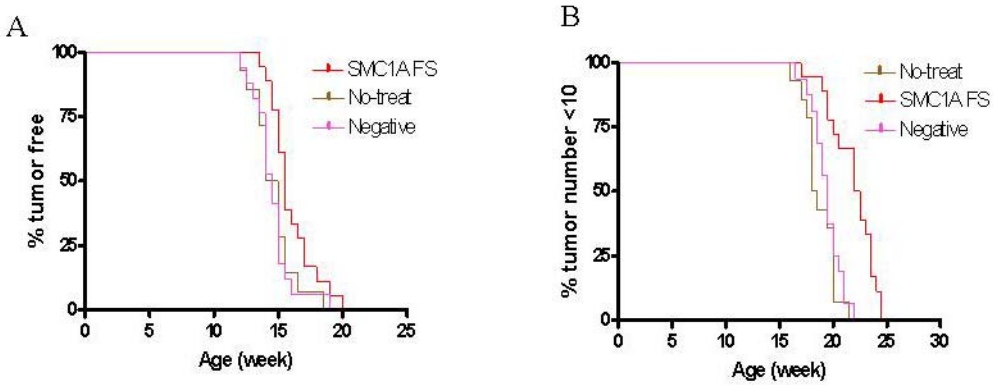


Figure2. 36 Tumor development in different immunization groups. A. Tumor free curve. P value of SMC1A vs. no-treated group and negative group<0.05. B. Tumor progression curve. Measure percentage of mice doesn't fully develop tumors. P value of SMC1A vs. no-treated group and negative group<0.0001; the SMC1A FS data were collected from two independent experiments with 11 mice and 7 mice respectively.

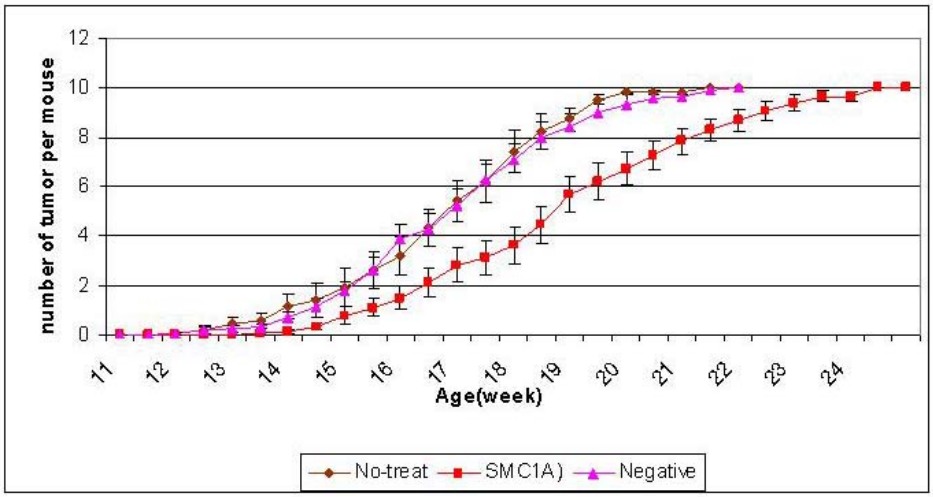


Figure2. 37 Tumor multiplicity curve of SMC1A group, negative group and no-treated group in BALB-NeuT model

There was no detectable IFN-gamma released splenocytes in these mice with different SMC1 FS antigens stimulation, including peptides, peptide conjugated to KLH (Figure 2.38). All mice had high IgG activity to SMC1A FS. IgG1 activity was the dominant activity and there were partial subtype switch to IgG2a and IgG2b (Figure 2.39). There was no detectable immune response to the wild type SMC1A peptide encoded by exon1 of the SMC1A gene including the IgG activity and the IFN-gamma released splenocytes. I further analyzed the relation between the IgG active and the tumor development in those protected mice (Figure 2.40). It indicated there was a significant positive correlation between the tumor progress time and the IgG and IgG1 activity to SMC1A FS peptide in first set of 11 mice.

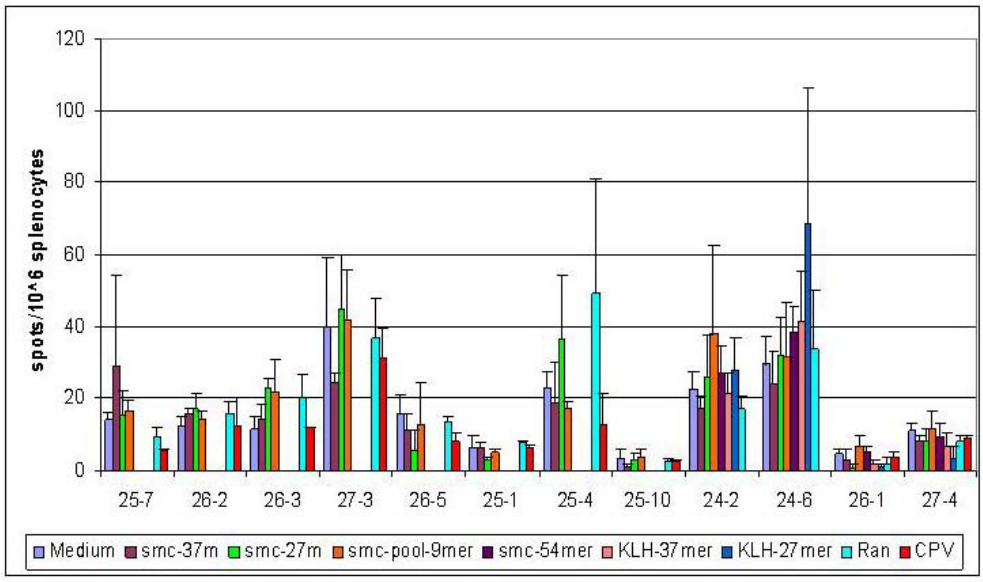


Figure2. 38 ELISPOT analysis of IFN-gamma release splenocyte in SMC1A immunized mice. X axis are individual mouse; stimulators: smc-37: WT(exon 1); smc-27: 10aa WT+17aaFS; Smc pool 9mer: 9mers in smc1-27. smc1-54mer: exon 1+FS. Ran: random peptide in genetic plasmid; CPV: pool peptides as negative control.

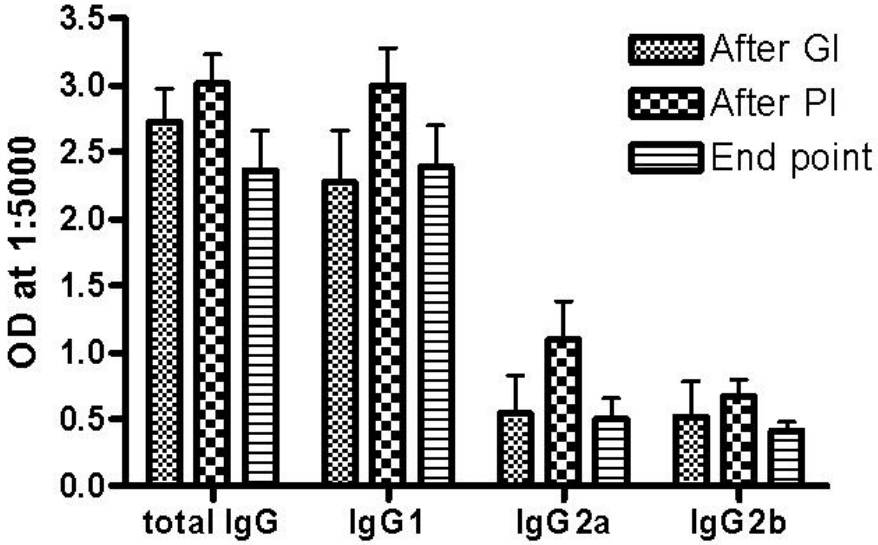


Figure2. 39 ELISA analysis of subtype IgG activity to SMC1A FS 27mer in SMC1A FS immunized mice (11mice). Each bar represents average OD of serum from 11 mice with duplicate at 1:5000 dilution; error bars are standard error.

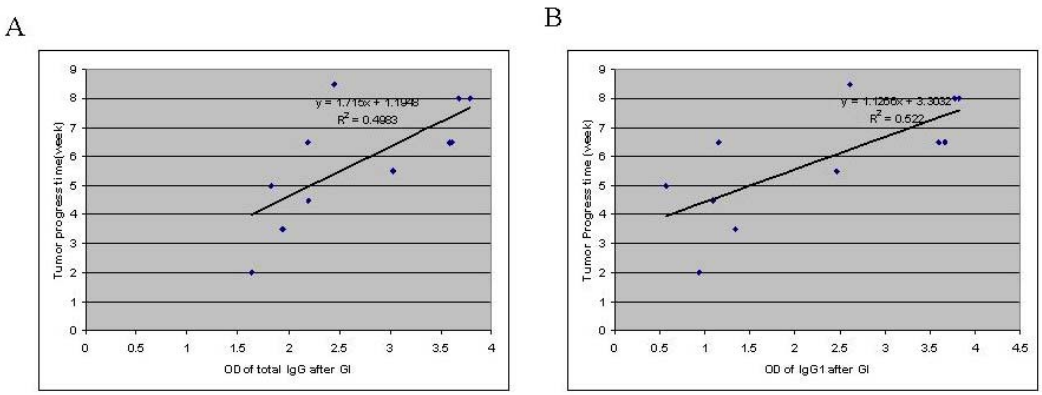


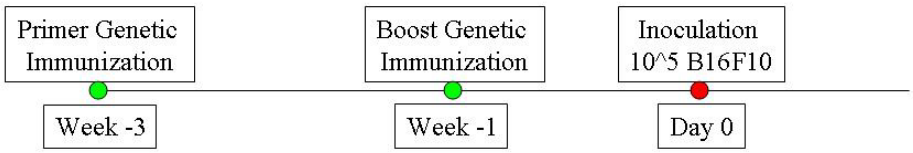
Figure2. 40 Correlation analysis of tumor progression time and IgG activity. OD of total IgG and IgG1 activity against SMC1A FS peptide were measured by ELISA of serum after last genetic immunization of 11 mice in first experiment. Tumor progression time was defined as time period of mouse getting first palpable tumor to 10 tumors. A. Correlation of total IgG activity to tumor progression time. B. Correlation of IgG1 activity to tumor progression time. Both p value <0.05 by linear regression analysis

2.2.2.3.3 Additive protection by combine other FS antigens

The FS antigens are usually short polypeptides containing limited epitopes compare to most currently identified self antigens. The expected additive protection from adding more FS antigens is one of the important anticipated characteristics of the prophylactic cancer vaccine. We expected pooling FS antigens to increase the efficiency of tumor prevention and provide more coverage of protection against different type of tumors. Single FS antigens as well as the combination of a few have already provided the protection in two mouse tumor models. Here I summarize some experiments I performed by directly comparing the protection of pooled and individual FS antigens.

First additive protection was analyzed by 1-78 and 6-21 immunization (Figure 2.41). With low FS antigen dosage and GMCSF as the adjuvant, both

individually immunized 1-78 and 6-21 groups had protection compared to the AAT control. Combining 1-78 and 6-21 improved the protection compared to the single FS immunization (Figure2.42).



Group Setting (10mice/group C57BL6) :

- Group1: AAT
- Group2: 1-78fs
- Group3: 6-21fs
- Group4: 1-78fs + 6-21fs

- Dosage: (dosage/mouse/immunization) 20ng/each antigen+1ug GMCSF
- 4 shot/mouse
- Inoculation: 1x10^5 B16F10/mouse

Figure2. 41 Detail experimental plan for additive protection of FS antigens in B16F10/C57BL6 mouse melanoma tumor model

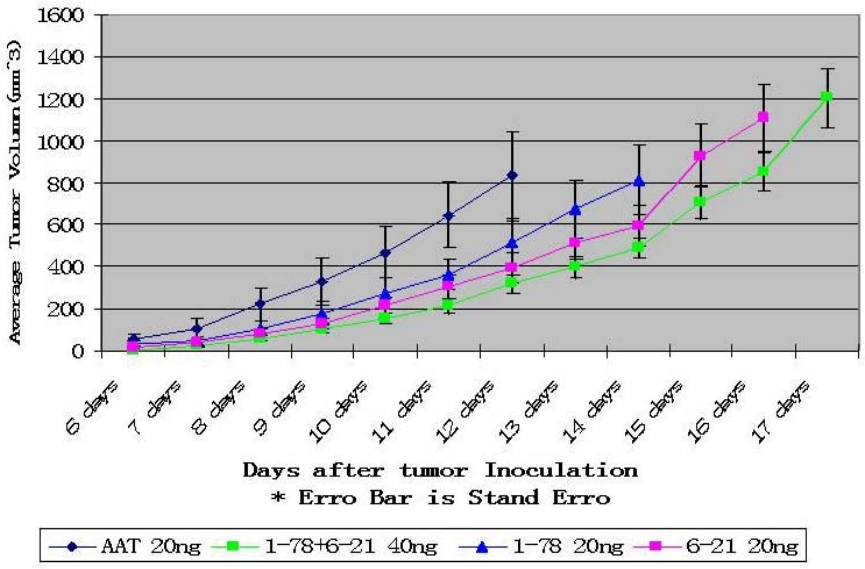
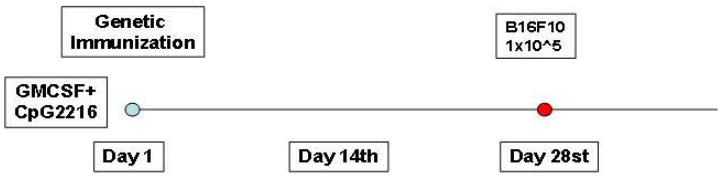


Figure2. 42 Tumor growth curve of different groups

The pool of 1-78 and 6-21 exhibited additive protection with the conventional genetic immunization. We expected to have stronger additive protection by adding SMC1A FS to the pool and the use of the new genetic immunization system and stronger combination adjuvant: CpG plus GMCSF. In the experiment, using combination of CpG and GMCSF as the adjuvant and one primary immunization (Figure 2.43), all individual FS antigens immunizations provided the inhibition of tumor development, as well as the pooled FS antigens immunization. However there was no improvement of the protection by pooling three FS antigens compared to individual ones (Figure 2.44).



Group Setting (10mice/group C57BL6) :

- Group1: CPV172 alone
- Group2: SMC1A FS
- Group3: 1-78fs
- Group4: 6-21fs
- Group5: 1-78fs+6-21fs+SMC1A FS

- Dosage: (dosage/mouse) 20ng/antigen+0.5ug GMCSF+5ugCpG2216
- 4 shot/mouse
- Inoculation: 1X10^5 B16F10/mouse

Figure2. 43 Detail experimental plan for additive protection of FS antigens in B16F10/C57BL6 mouse melanoma tumor model

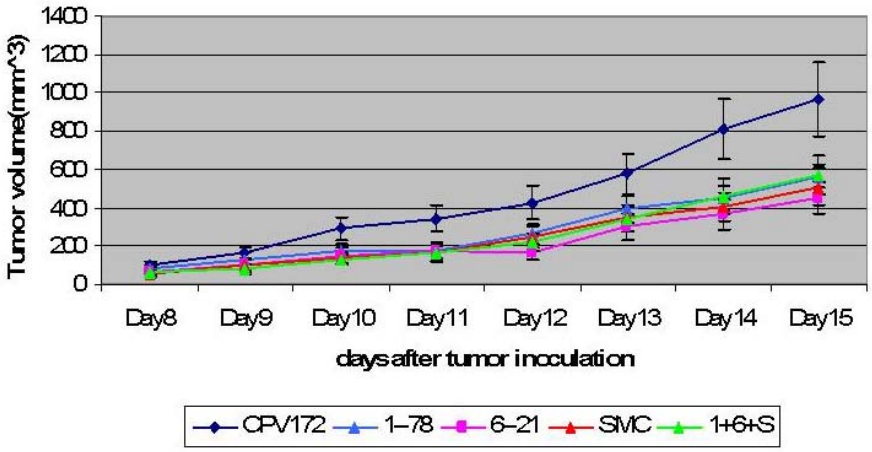


Figure2. 44 Tumor growth curve of different groups

Comparing the tumor growth curve of the pooled FS antigens group to an earlier experiment with the same adjuvant and similar antigens setting, the protection was reduced in the later experiment (Figure 2.45). The different protection efficiency between the two experiments may be caused by different preparation of the micro gold particles for the genetic immunization (Figure 2.46). In the earlier experiment, each gold particle were coated with pooled antigens, consequently, each active DC could present all these antigens; while in the later experiment, each gold particle only contained a single FS antigen, consequently, each active DC could only present one antigen. We need to further investigate if the different preparation methods cause the different efficiency of immune response and consequently cause the different efficacy of the protection. Earlier experiments in infectious disease models had shown that preparing gene gun bullets that carried plasmids encoding a mixture of antigens on each microparticle performed better than attaching plasmids encoding only one antigen per

microparticle. (Kathryn and Bert ASFV manuscript in preparation, and Dr. Stephen Johnston, personal communications).

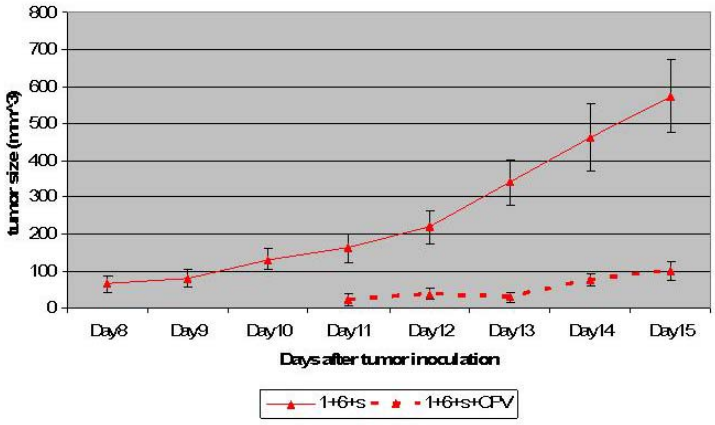


Figure2. 45 Comparison of tumor growth curves from different experiment with the same immunization regimen. 1+6+s group was immunized combination of 1-78fs, 6-21fs and SMC1A FS; 1+6+s+CPV group was immunized with the same combination of FS antigens plus CPV172. They all used the same immunization regimen as described in Fig 1.54. Error bars are standard error

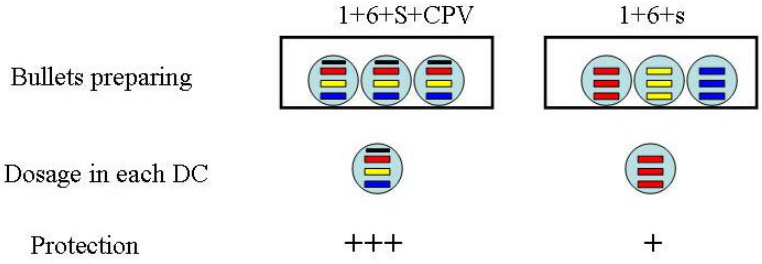


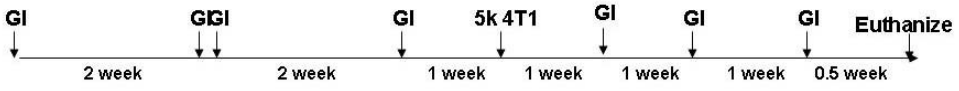
Figure2. 46 Comparison of the bullets preparation of two individual experiments. In 1+6+s+CPV group immunization, the gold particles carried pooled antigens; while in 1+6+s group immunization, the gold particles carried individual antigen.

The additive protection was also observed in genetically modified mouse tumor model. Combination of the effective FS antigens SMC1 and 1-78 and

negative antigens 6-21 and CPV172 had significant improvement of protection in the FVB/N-NeuT model (Figure 2.30).

2.2.2.4 Evaluation of metastasis prevention

The immune response analysis of SMC1A FS immunization demonstrated that the tumor development can boost the specific immune response by increasing the T cell activity (Figure 2.21). The protection analysis in transgenic mouse models also revealed that the established specific immune response to FS antigen not only could prevent the first tumor initiation, but also could more efficiently prevent the second tumor and later independent tumor initiations (Figure 2.37). All of these results indicated that the tumor development could boost the memory immune response which was elicited by the prophylactic immunization. With the proper prophylactic immunization, the tumor cell could enhance the immune response to the antigens in the prophylactic vaccine. This implies that a prophylactic cancer vaccine could not only inhibit the primary tumor, but could also prevent or inhibit tumor metastasis.



Group Setting (10mice/group BALB/c)
- Group1: empty plasmids
- Group2: SMC1A FS
- Group3: no-treated

- Dosage: (dosage/mouse/immunization) 1ug/antigen+0.5ug LTAB+5ugCpG2216
- 4 shot/mouse
- Inoculation: 5X10^3 4T1 cell/mouse

Figure2. 47 Detail experimental plan for anti-metastasis evaluation of SMC1A FS antigen in 4T1/BALB/c mouse breast tumor model

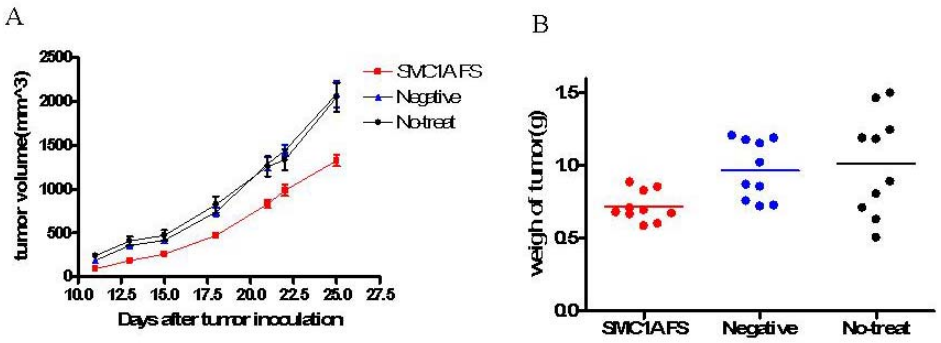


Figure2. 48 Protection evaluation against primary tumor development. A. Tumor growth curve of different groups. P value of SMC1A FS vs. negative or no-treated group <0.05; B. Endpoint tumor weight of individual mouse. SMC1A FS antigen in 4T1/BALB/c mouse breast tumor model. P value of SMC1A FS vs. negative or no-treated group <0.05

For the preliminary experiment, I evaluated lung metastasis prevention in the 4T1/BALB/c model by the prophylactic immunization of SMC1A FS (Figure 2.47). Besides the SMC1A FS immunization group, there were two control groups: non-treated group and negative control group with the empty plasmids immunization. The experiment was designed to try to reflect the real tumor treatment based on immune therapy (Figure 2.47). 4T1 tumor cells were inoculated after the prophylactic immunization. Three weekly immunization boosts were applied after palpable tumor detection to strengthen the immune response. The count of 6-thioguanine resistant 4T1 cell colonies from the whole lung cells in *in vitro* culture dishes was used to evaluate the efficacy of metastasis prevention. The SMC1A FS immunization successfully inhibited the primary tumor development comparing to the negative or the no-treatment group (Figure

2.48). Though there may have been a trend for reduced metastasis colonies in the SMC1A FS vaccinated group, the differences were not significant (Figure 2.49). Over all, the primary tumor size was positively correlated with the lung metastasis colon number (Figure 2.50). This corresponded with the literature reports.

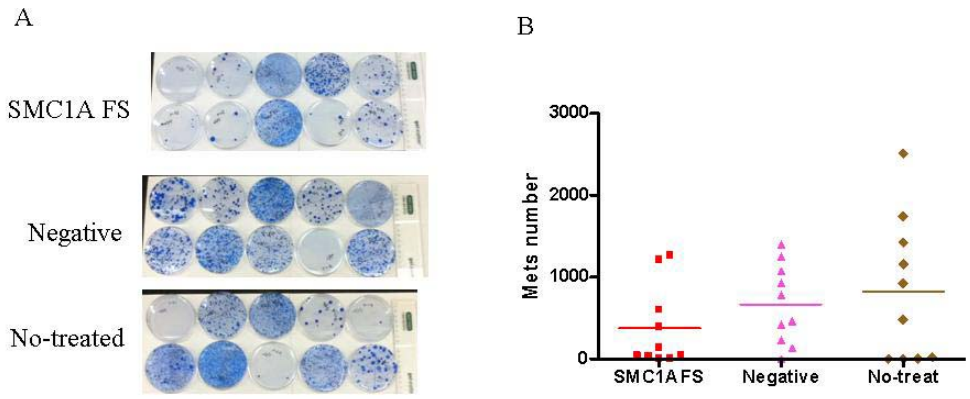


Figure2. 49 4T1 lung metastasis assay. A. pictures of the 4T1 lung metastasis clones. Whole lung from each mouse was harvested and digested into single cells after authorization of the mouse. All cells were resuspended in culture media with 6-thioguanine and incubated for 14 days. The 4T1 clones were fixed and stained with methanol blue. B. Count clone number of each lung. p value of SMC1A FS vs. negative or no-treated group>0.05

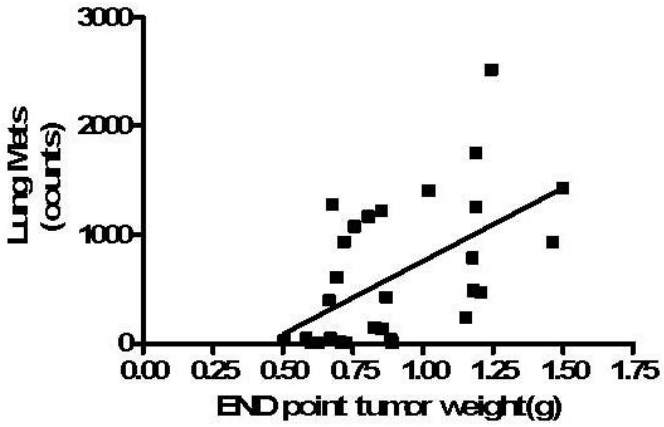


Figure2. 50 Correlation analysis of over all tumor size and lung metastasis colon number. Linear regression analysis p<0.005

Although we did not significantly inhibited the 4T1 metastasis in this experiment; the trend of the inhibition by SMC1A FS immunization is still encouraging. The optimization of the immunization regimen and including more FS antigens should help efficiently inhibit the 4T1 metastasis. Also this experiment did not directly test the concept of the prophylactic metastatic cancer vaccine as we vaccinated after tumor developed. So we could not separate therapeutic effects. The multiple immunizations could also have an immune inhibiting effect.

2.2.3 Prevention of tumor development by innate stimulations of the immune response

The prevention of tumor development by innate stimulation of the immune response is well documented in both various animal models and clinical trials. I also observed tumor prevention by influenza infection in the BALB-NeuT model. The experiment was designed for immunosignature analysis of chronic disease (cancer) and acute infection (influenza). All mice were infected with a non-lethal dose of influenza strain A/PR/8/34 at 6 weeks old or 10 weeks old. They all successfully recovered in 2 weeks after the infection. Although compared to the no-treatment group and the group infected at 10 weeks old, there may have been trend of tumor inhibition in the group infected at 6 weeks old, the difference was not significant (Figure 2.51). The influenza infection could strongly induce the innate immune response through Toll-like receptor (TLR), the retinoic acid inducible gene I (RIG-I) and NOD-like receptors (NLR) [174]. The active

innate immune response release proinflammatory cytokines and type I interferons to against virus infection. It also sensitizes innate immune surveillance to inhibit the tumor development. Because of the continuous expression of the strong oncogene in this mouse model, the sensitized innate immune surveillance by influenza infection may not sufficient to inhibit the tumor development.

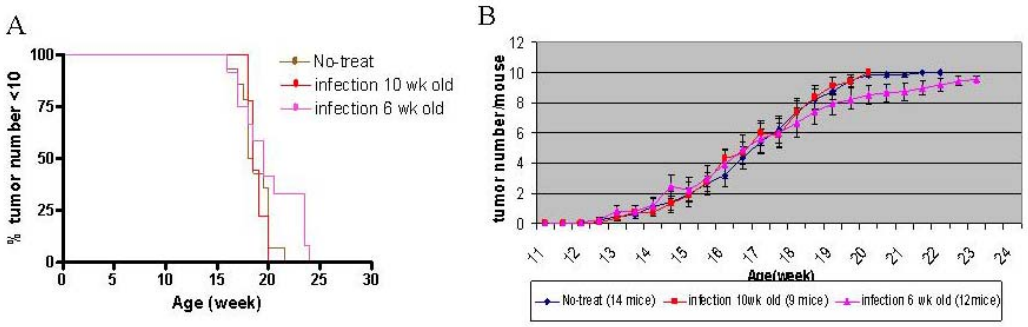


Figure2. 51 Influenza infection of BALB-NeuT mice. Two group of mice were infected with non-lethal dosage of influenza strain A/PR/8/34 at 6 weeks old (n=12) or 10 weeks old (n=9). A. Tumor progression curve; B. tumor multiplicity curve. No significant different among three groups.

Based on the previous experiments, I tested two different innate stimulators in the BALB-NeuT model: CpG2216 plus GMCSF and B2L. The CpG2216 plus GMCSF were delivered by the gene gun with the synthetic ODNs and the plasmid expressing GMCSF. All mice were given five treatments in 3 week intervals from5 to 6 weeks old (Figure 2.52 A). However, this could only partially inhibit the tumor progression without significant difference compared to non-treated group (Figure 2.52 B and C).

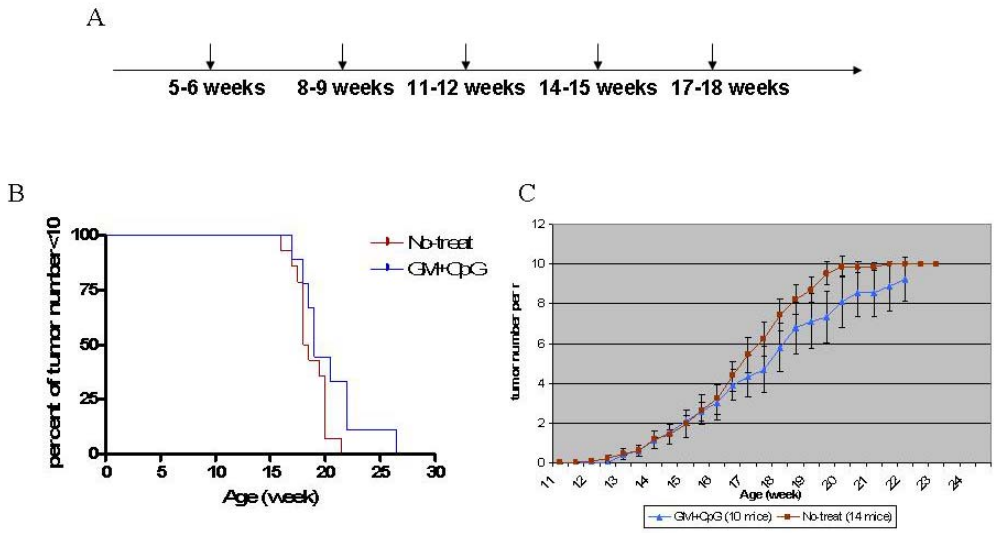


Figure2. 52 CpG plus GMCSF treatment of BALB-NeuT breast tumor model. A. treatment schedule, all mice treated with 5ug CpG2216 plus 1ug GMCSF each time through gene gun. B. Tumor progression curve. No significant different from no-treatment group. C. Tumor multiplicity curve. No significant from no-treatment group.

In B2L treatment experiment, the treatment of empty plasmid served as the negative control of B2L treatment. The schedule of the treatment was the same as the CpG plus GMCSF treatment (Figure 2.53 A). The B2L treatment did not significantly inhibit the first tumor initiation. However the B2L treatment did significantly inhibit the tumor progression (Figure 2.54 B and C). Further investigations need to be performed in analysis of protection mechanism as well as the treatment optimization.

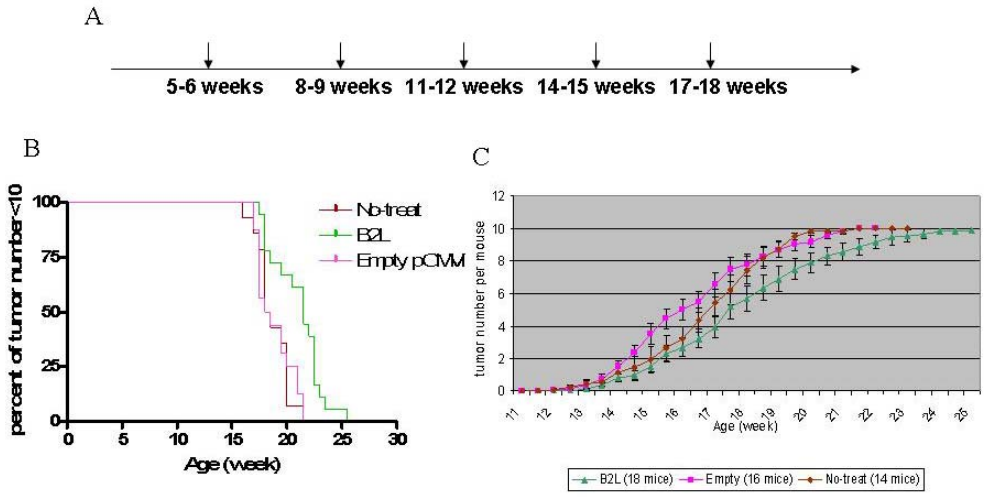


Figure2. 53 B2L treatment of BALB-NeuT mouse breast tumor model. A. Treatment schedule. all mice were treated 2ug B2L (n=18), empty plasmid (n=16) or no-treated (n=14) each treatment was with the gene gun. B. Tumor progression curve. P value of B2L vs. empty plasmid group and no-treatment group <0.001. C. Tumor multiplicity curve.

2.3 Materials and Methods

2.3.1 Mice

Four to five weeks old wild type BALB/C and C57BL6 mice were ordered from Chariles River Laboratories International, Inc. BALB-NeuT and FVB/N-NeuT mice were bred at ASU. Thanks to Dr. Joseph Lustgarten at Mayo Clinic Arizona for the breeding pairs. All mouse related studies were under the animal use protocol 1000R and revised to 1197R. Animal use protocols were approved by Arizona State University Animal Care and Use Committee.

2.3.2 Cell lines.

All the cell lines were purchased from ATCC and cultured under the ATCC recommended media and conditions. HPDE 6 was kindly send by Dr. Douglas Lake.

2.3.3 Tumor samples and cDNAs samples

All dog cDNAs were supplied by Dr. Doug Thamm, Colorado State University. Pancreas Cancer patient matched tumors and normal adjacent tissues were offered by Dr. Douglas Lake. Human primary breast tumor cDNAs and normal mammary gland cDNAs were purchased from BioChain Institute, Inc.

2.3.4 RT-PCR

Total RNA was extracted with TRIzol and the RNA purification kit (Life Technologies, Carlsbad, CA) following the manufacturer protocols. Total RNA concentration was measure by Nano-drop 1000 (NanoDrop Products, Wilmington, DE). cDNA was synthesized from 1ug total RNA with the SuperScript^{TM} III First-Stand Synthesis SuperMix (Life Technologies, Carlsbad, CA) following the manufacturer protocols. All the primers were designed using Primer3 or GeneRunner and synthesized by Life Technologies, Carlsbad, CA or Sigma. End point PCR was performed with Gotaq^{TM} PCR kit (Promega Corporation, Madison, WI) and Mastercycler ep gradient S (Eppendorf, Hamburg, Germany).

2.3.5 Quantitative PCR

The quantitative PCR with Taqman™ probe was followed with the manufacturer protocol.

SMC1A FS probe: CAATGGCTCTGGGTGCTGTGGAATC

SMC1A FW: 5'- GGGTCGACAGATTATCGGACC

SMC1A RV: 5'- GTCATACTCCTGCGCCAGCT

2.3.6 ELISA

The following is the standard protocol I used for all of the ELISA assays in this thesis. ELISA plates were coated with 50 uL of 10 ug/mL of peptide or protein in carbonate coating buffer and incubated at 4° C overnight. The coated plates were washed 3X with PBST and blocked with 200 uL of 3% BSA in PBST at 37° C for 30 minutes. The blocked plate was washed 3X with PBST and 50 uL of primary α-serum or purified antibody diluted in 3% BSA in PBST was applied. The plate was then incubated at 37° C for 1 hr. After the incubation, the plate was washed 3X with PBST. The antibody was detected with 50 uL HRP-goat α-rabbit IgG diluted 1:2000 in 3% BSA in PBST. After the plate was incubated at 37° C for 1 hr, the plate was washed 3X and developed with 50 uL TMB for 10 minutes at room temperature. The development was stopped by adding 50 uL of 0.5 N HCL, and the plate was read with a SpectraMax 190 Molecular Devices instrument at OD 450 nm.

2.3.7 IFN-γ ELISPOT

ELISPOT assays were performed with mouse IFN-γ ELIPOT sets (BD Biosciences, San Jose, CA) following the manufacturer protocols. The plate was coated IFN-γ capture antibody according the protocol at 4°C over night and washed two times with complete RPMI medium before blocked with complete RPMI medium at 37°C for 2 hours. 10^6 mouse splenocyts were then plated in each well and co-cultured with 20ug peptide or protein and a final volume 200ul RPMI complete medium for 48 hours. Each peptide or protein was assayed in triplicate. After cell activation, the plate was incubated with biotin labeled IFN-γ detection antibody, HRP labeled strepavidin and final developed with EC substrate following the protocols at room temperature for 15 minutes. The plate development was stopped by washing wells with DI water and air-drying at least for 2 hours.

2.3.8 Genetic Immunization

Mouse genetic immunization was performed with the Helios® Gene Gun System (Life Science Research, Hercules, CA) by published protocols [143-146].

Conventional bullets preparation

The gold is weighted based on the calculation (0.5mg of gold is needed per bullet), and washed with 1x water, 1x 95% ethanol and 2x water. Gold is resuspended in calculated DNA solution and add equal amount $CaCl_2$. 10% total volume of 1M spermadine is added drop by drop and vortex on ice for 30

minutes. The gold is spun down and washed in three times with 100% ethanol. The gold is resuspended with 0.05x PVP solution and transfered into the tubing. The mixture is allows to sti for 15minutes, then discarded and the tube dred with Helium for 20min. The tubes are cut and stored in 15ml tubes with dryrite in the bottom.

Charged gold preparation

2 grams gold are washed with15ml H_2SO_4 and 5ml 30% H_2O_2. After the solution cools to room temperature, wash the gold with 8 times 40ml water and 2 times 20ml 100% ethanol. Resuspend the gold with another 20ml ethanol and add 0.5g 11-mercaptoundecanoic acid. Shake the mix at 1400 rpm for 2 hours at room temperature. After vortex, wash the gold 2 times with 40ml water. Resuspend the gold in 20ml 0.1M MES, add 300mg NHS and 200mg EDC directly into the mix and keep shaking at 1400rpm for 30 minutes. Remove the supernatant after the incubation, add 20ml PEI1750 (2 grams in 20ml water and pH9) and keep shaking another 2 hours. Finally, wash the gold 2 times with 40ml water and lyophilize overnight. The dried gold should keep at 4 °C.

Bullets with charged gold

Make 167mg/ml charged gold in 0.1M MES [2(N-Morpholino)ethanesulfonic acid] solution (pH6). Add the calculated amount of charged gold (6ul per bullet) to the DNA solution and vortex 10 minutes. Then spin down the gold-DNA complex and wash with 1ml ethanol. Resuspend the washed complex with butanol (55ul per sample), and let sit still for 15 minutes.

Discard the butanol and dry with helium for 20 minutes. Then cut thetubes and store in 15ml tube with dryrite.

Bullets with nanogold particles

Nanogold particles preparation: add 25mg $HAuCl_4$ to 40ml water and mix well. Then add 400ul 213mM fresh cysteamine and stir with a spinbar for 10 minutes. Add 10ul 10mM sodium borohydrate and keep stir for 30 minutes. The final solution should be deep wine red.

Bullets preparation: add calculated DNA into 1ml nanogold solution, vortex for 10 minutes. Then add calculated charged gold (167mg/ml charged gold in 0.1M MES, and 6ul per bullet) into the mixed solution, Mix occasionally at RT for 10min. Spin down the gold complex, wash with 1ml ethanol. Resuspend the washed complex with butanol (55ul per sample), and let sit for 15 minutes. Discard the butanol and dry with helium for 20 minutes. Then cut the tubes and store in 15ml tube with dryrite.

2.3.9 4T1 lung metastasis assay

The 4T1 lung metastasis assay was followed the protocol proposed by Pulaski [238]**.** Briefly, the lung was taken from euthanized mice, minced into small pieces and digested in final volume 5 ml collagenase type IV/elastase cocktail for 75 minutes at 4°C. Filter the digested samples through 70um nylon cell strainer and wash with 10ml 1xHBSS 3 times. The cell pellet was resuspended with 10ml IMDM supplemented with 10% FBS, 1×antibiotic-antimycotic (Life Technologies), and 60 μM 6-thioguanine and plated into a

10cm cell culture dish. Place dishes in a 37°C tissue culture incubator, 5% CO_2 for 10-14 days. After the incubation, the cells were fixed with 5ml methanol and stained with 5ml 0.03% methylene blue. The colonies were counted after the plate dried.

2.4 Discussion

Although different types of tumors possess different FS transcript profiles, the whole list of FS candidates is enough to cover the tumor types that we have investigated so far. For example, the SMC1A FS transcript was detected in almost all of the different human tumor cDNA samples we investigated, including breast (n=34), pancreas (n=4), and lung cancer (n=1) cell lines and primary breast (n=7) and pancreatic tumors (n=4). We also detected homologous SMC1A FS transcripts in all mouse and dog tumor samples, including melanoma cell lines (n=2), one breast tumor cell line, primary tumors of three spontaneous mouse breast tumor models and 22 cDNAs from 8 different primary dog tumors, such as melanoma, osteosarcoma, lymphosarcoma, breast tumor etc. I also detected other FS transcripts in most of these cDNAs, such as 6-21 and 1-78. Detection of SMC1A FS transcript in the early stages of the FVB/N-NeuT breast tumor indicated that some of these FS transcripts may be involved in tumor development and could be exposed to the immune system at the early stages of tumor development. Those FS antigens are the most valuable candidates for a prophylactic cancer vaccine development which tries to prevent tumor at early stage. The high frequency of FS transcripts that were confirmed by

screening of a broad tumor sample setting and the high coverage of HLA types by *in silica* analysis suggest that a general prophylactic cancer vaccine is feasible (Lee and Johnston, in preparation). Further investigation in more tumor samples is necessary to have a more accurate estimation of FS frequency in different human cancers.

With the limitation of the proteomics analysis, I did not confirm the translation of SMC1A FS transcript in tumor cells. The western blot, IHC and IF analysis implied that the SMC1A FS protein may be expressed and SUMOylated in both tumor cells and normal tissues (see detail at Chapter 4). In any case, the absence of the detectable FS protein is a common situation in this research area. For instance, only 2 of 18 FS mutations found in the MSI-high colon tumor cell lines were detected at the protein level by western blot and confirmed by siRNA knock down [169]. This indicates that the impaired product quality control systems in tumor cell, such as NMD and ERAD, can still efficiently decrease the translation of some FS antigens and keep them below the limitations of the current direct detection technologies. The absence of direct evidence of translation FS antigens does not indicate that they are not suitable targets for vaccine development. The NMD is the translation dependent process. The degradation of NMD targeted transcripts must be trigged by the primary translation [175]. Therefore, the NMD targeted FS transcripts may possibly be translated even without the detection of the transcripts. Consequently, these transcripts can still be presented and detected by the immune system, which is

more sensitive and competent detection system than any other current technology [176]. Researchers have shown that a few target peptide/MHC complexes are sufficient to trigger specific CTL mediated killing [177-180]. This indicates that all of these FS antigens still could be good vaccine candidates by eliciting specific and efficient immune response. There are several reports supporting this idea. For example, the FS mutation of TGFBR2 occurs in 80-90% of the colorectal cancers (CRCs) with MSI. This mutant gene encodes a truncated protein bearing a 49 amino acids FS peptide if there is a nucleotide deletion in the cMS region. The FS transcript of TGFBR2 is sensitive to NMD in the MSI colon cancer cell lines. No FS truncated protein of TGEBR2 was directly detected [147]. On the other hand, researchers successfully detected the immune responses that are specific to the TGENR2 FS peptides in CRCs, including specific IgG activity in 10% of the CRCs and specific IFN-gamma released T cell in about 50% of the CRCs in two independent studies [181, 182].

This may be the same situation for the SMC1A FS truncated protein. I successfully detected the specific immune response of both antibody and T cell to the SMC1A FS peptide in tumor bearing FVB/N-NeuT mice. That is direct evidence of translation of the SMC1A FS antigen in tumor cells. These results also indicated the immunogenicity of SMC1A FS antigen and that it is an effective candidate for vaccine development.

The limitation of current technologies, especially the technologies in proteomics analysis make them inefficient methods for cancer vaccine candidate

screens. I used the mouse tumor model system to efficiently screen the prophylactic cancer vaccine candidates by directly evaluating the tumor prevention caused by FS antigens in the tumor models with the optimized immunization regimen. This approach can evaluate all factors for a vaccine candidate in one sensitive system, such as expression, immunogenicity and protection. With this system, we first demonstrated the concept of the prophylactic cancer vaccine with FS antigens based on the analysis of the SMC1A FS antigen and/or other FS antigens. The additive protection with variable FS antigens compositions in different tumor models further supports our concept. Although the SMC1A FS antigen is only 27 amino acids long, and contains only a handful of predicted epitopes with low binding scores to all MHC I and MHC II haplotypes in both BALB/c and C57BL6, the immunization of SMC1A FS antigen in the prophylactic setting successfully produced significant inhibition of primary tumor growth of mouse melanoma (B16F10/C57BL6), mouse breast tumor (4T1/BALB/c), as well as two transgenic spontaneous mouse breast tumor models (FVB/N-NeuT and BALB-NeuT), which may closely mimic in some respects the natural development of human breast cancer. The immune response analysis revealed that although SMC1A FS is a poor immunogenicity antigen by *in silico* analysis, both specific memory humoral and cellular responses are successfully elicited and correlated with tumor inhibition in different mouse models. In C57BL6 mice, the genetic immunization of SMC1A FS antigen elicited memory cellular immune response which was efficiently stimulated after

B16F10 tumor cell inoculation with more than 20 fold higher activity and significantly inhibited primary tumor growth. The antibody activity is variable depend on different immunization regimens. In BALB/c mice, the primary 4T1 tumor was significantly inhibited even when the tumor cell were inoculated more than 25 weeks after a single low dosage genetic immunization of a pool of 3 FS antigens and a negative control antigen. The same immunization regimen in 4 week old FVB/N-NeuT mice with a single SMC1A FS antigen or a pooled antigens inhibited spontaneous breast tumor development. This indicated that there was an effective memory immune response that was elicited in both mouse models. The end point mice with no detectable immune response may not have had a strong response due to systemic immune suppression by fully developed tumors. The tumor inhibition in the negative control group in FVB/N-NeuT indicated the antitumor activity of the adjuvant included in the immunization. Further immunization optimization is necessary to clarify the correlation of immune responses and individual FS protection. The SMC1A FS has shown significant protection in the BALB-NeuT mouse tumor model with an optimized immune regimen. The tumor inhibition is correlated with specific IgG activity to the SMC1A FS peptide.

The analysis of protection in transgenic mouse models demonstrates that the prophylactic cancer vaccine can more efficiently inhibit multiple tumor development after the first tumor has been established. This indicates that the tumor itself can boost immune surveillance at the prophylactic immunization. The

immune response analysis in the B16F10/C57BL6 mouse model supports this hypothesis. The specific cellular immune response was boosted 20 fold higher by tumor cell inoculation. This led us to develop the concept of prophylactic cancer vaccine against tumor metastasis. The metastasis of 4T1 tumor cells usually starts around 7 days after the primary tumor inoculation. This leaves a short time window for the immune system to have been boosted by the primary tumor. This may be the reason that there was no significant metastasis reduction by the SMC1A FS immunization alone. These results may also have been caused by an insufficient immune response elicited by the current immunization regimen and antigen composition. These factors should be tested in further experiments with another proper metastasis model with a longer metastasis window, which more closely resembles natural tumor metastasis development.

Our results and other studies demonstrated the expansion of the pre-activated tumor specific adaptive immune response could be augmented by the primary tumor itself [183]. This tumor activation of vaccine immune response could not only inhibit the primary tumor development, but also have potential to inhibit or prevent tumor metastasis, which I tested in my study. Additionally, this tumor derived immune response activation led me to development a new concept for cancer early detection: “amplified diagnosis”. The concept is straightforward: healthy people receive prophylactic immunization with a set of tumor specific antigens to establish an efficient memory immune response. The memory immune response will be activated by exposure of the immune system to some of these

antigens being presented by the tumors. This response would presumably be stronger and earlier than the endogenous immune response without pre-vaccination. By monitoring the specific immune response, such as antibody activities against the immunized antigens, we can detect early tumor onset. As I discussed in the 1st chapter, people are exploring early cancer diagnosis with the tumor derived autoantibody detection. However, the endogenous adaptive anti-tumor response is always markedly delayed. For example, about 45% of colon cancer patients have p53 mutations, and about 50% of colon cancer patients have P53 over-expression [184, 185]. Johannnes et. al. reported that with the overlapping P53 peptides array, they could detect anti-P53 autoantibody about 1.4 years (median lead time, range 0.12-3.8 years) prior to clinical diagnosis of colon cancer [186]. It usually takes about 40 to 50 years for a colon tumor developing a clinically detectable size. We believe that with "amplified diagnosis", we should detect these tumor specific immune response much earlier. Actually, the concept of the amplify diagnosis could be use for early detection of any disease with specific immune response. We should further test this concept, especially in cancer early diagnosis.

The autoimmunity caused by the off target effects of a cancer vaccine is always a major concern, especially in prophylactic cancer vaccine development. Actually, with the proper administrations, almost all clinical cancer vaccine trials with cancer associated self antigens have been remarkably safe. The slight autoimmunity symptoms in some trials were tolerable and are related to a better

treatment outcome. There is no evidence of severe autoimmune activity related to a specific immune response to those self antigens in all therapeutic clinical trials and even in animal trials with a prophylactic immunization setting.

As described previously, compared to self antigens, the cancer specific and associated FS antigens are a better source for a prophylactic cancer vaccine development in both effectiveness and safety. The fully functioning quality control systems and other mechanisms in normal cells prevent FS antigens from been produced and displayed to the immune system, and this avoids the off target effects. Although there are no thorough studies about the safety of prophylactic cancer vaccines based on FS antigens in clinical trials yet, the results from related clinical studies indicate the safety of the prophylactic immunization of FS antigens. For instance, both the humoral and cellular immune response to specific FS antigens were detected in lynch syndrome patients with the MSI CRC and the healthy non-tumor bearing individuals [181]. The lynch syndrome is caused by inherited mutations in DNA mismatch repair genes, which can subsequently induce the MSI and increase the risk of colon cancer and other related cancers. There is no report about serve autoimmune diseases in those healthy lynch syndrome patients. On the contrary, compared to the sporadic CRC, the better outcome of MSI CRC with lynch syndrome is correlated with a higher frequency of tumor infiltrating lymphocytes. This indicates that the immune responses against FS antigens will not cause server autoimmune diseases. On the contrary, they could contribute to tumor inhibition.

As I discussed in the prophylactic cancer development, there are three levels of control systems to ensure that the FS antigens are specific to tumor cell: genetic control, transcription control and translation control. The most specific FS antigens are generated by the genetic mutations in tumor cells. Apparently, the SMC1A FS antigen is not the most specific FS antigen. SMC1A FS transcript was detected in some normal cDNAs from variable organs, such as cDNA from normal mouse splenocytes displaying similar levels of the FS transcripts as B16F10 tumor cells. This may be caused by the alternative NMD in those immune cells as indicated by other studies [137, 187]. And the western blot, IHC and IF analysis also implied that the SMC1A FS protein may be expressed and SUMOylated in both tumor cells and normal tissues (see detail at Chapter 4).

However, the results from my studies implied that the SMC1A FS antigen is still safe for prophylactic immunization. With a detectable SMC1A FS transcript level in a variety of normal cells, even in normal immune cells, there is no detectable specific immune response to the SMC1A FS antigen in normal mice. This clearly indicates that normal cells have full control to avoid the SMC1A FS antigen been displayed to and recognized by the immune system. There may be two mechanisms for the specific control. First, most normal tissues have low level or no detectable level of the SMC1A FS transcript, so the expression of the SMC1A FS protein has been tightly controlled by the NMD and ERAD in these cells. Second, except the normal heart tissue, only the SUMOylated form of the SMC1A FS was detectable in the normal tissues (see

details in Chapter 4). The SUMOylation tightly controlled the subcellular localization of the SMC1A FS protein and prevented it from being presented. Another directive evidence of the safe of the SMC1A FS antigen is that there are no severe autoimmune reactions observed in the mice with various intense immunizations of SMC1A FS and other FS antigens in my studies. Further complementary analysis is necessary to clarify the safety of FS antigens.

The investigation of tumor inhibition with innate stimulation demonstrated that the tumor can be inhibited by utilizing only innate stimulation. I tested the concept of a prophylactic innate stimulation in the BALB-NeuT mouse tumor model. In the preliminary data, the prophylactic administration of B2L could significantly prevent spontaneous breast tumor development in the mouse model. GMCSF and CpG had similar effects. Further investigation is necessary to optimize the administration and characterize the mechanisms of the tumor inhibition.

In summary, I believe my studies, as well as others in the Center, support the idea and suggest strategies for pursuing tumor prevention through a prophylactic cancer vaccine and prophylactic innate stimulation. Further investigation will focus on optimization of the administration, identification of more components and integration of different strategies.

CHAPTER 3

CANCER DETECTION

3.1 Introduction

Cancer detection is one of the major components for the global cancer control. The development of the cancer detection technology has significantly contributed decreasing mortality decreasing in several major cancer types. The serological detection of cancer has unique advantages in sensitivity, specificity and simplicity.

Based on our investigations of tumor FS antigens, I discover two new potential serological cancer biomarkers: the antibodies against FS peptides and the FS transcripts. They not only share the advantages of current autoantibody and nucleotide biomarkers, such as signal amplification, but also have advantages of the specificity by their unique characteristics. I also developed a unique detection strategy for each of them which make them more sensitive and robust. Here I will discuss the principle studies of these two cancer biomarkers for early cancer detection.

3.2 FS antibody as cancer biomarker

3.2.1 Introduction

As discussed previously, the NMD and ERAD and other quality control systems of normal cells reduces the incidence of the elicitation of antibodies against FS peptides by normal cells. This unique characteristic of the FS

peptides increases the specificity of the FS-antibodies as cancer biomarkers. As discussed, the FS peptides are mutant peptides, and should be novel to the immune system. This makes FS peptides more sensitive at eliciting the specific antibody reaction in vivo, unlike the autoantibody response to the self-antigens that are tolerated by the immune system.

Besides the sensitivity and specificity, there is another important characteristic that needs to be addressed before using the antibodies as biomarkers: the antibodies that are elicited in vivo are highly diverse in two aspects. First, the in vivo elicited antibodies to a specific antigen are always polyclonal antibodies. There are multiple B cell clones that could be activated even by a single epitpoe. Second, the antibodies in the whole population are more divers because of various genetic backgrounds of the immune system in the population, such as various HLA types. This high diversity allows the antibodies to recognize all of the possible epitopes of a specific antigen, including the linear epitopes and conformational epitopes. A method designed to robustly detect these antibodies in the population should consider the diversity of the antibody. However, most current autoantibody biomarkers target big antigens, such as P53 which is 393 amino acids long and naturally folds into a 3D structure. The current strategies of autoantibody detection are either using the recombinant proteins or overlapping peptides. Those recombinant proteins are folded and can not fully represent all linear eptitopes, while the overlapped peptides can not represent the conformational epitopes. Therefore, the current strategies for antibody detection

will lose part of the antibody signal either way and consequently cause a decrease in sensitivity.

Although most FS mutations usually generate short and simple FS peptides, they still contain multiple epitopes. Therefore the antibodies in the population that could recognize the FS peptides are still quite diverse. The efficient epitope representation of the FS peptide is still necessary. However, because of the uncontrolled orientation of the peptides on the surface and the space limitation, the natural peptides tend to not perform as well on a surface as in solution. To avoid these limitations, I developed a unique strategy to more efficiently exposure the epitopes of the FS peptides by using a set of mimotopes. There are three advantages: First, with a set of mimotope, the possible epitopes of the FS peptide can be fully represented. Second, these mimotopes are screened using the random peptide arrays and selected to function on the surface. Therefore, all of the epitopes can be fully represented and accessible to all of the FS-antibodies. Third, sometimes mimotope can elicit a better immune response than the original peptide [96]. This indicates that the mimotope could have higher affinity to antibodies than the original peptide depending on HLA backgrounds. The same strategy can also be used for the autoantibody detection. To prove the concept, I used SMC1A FS peptide as the target for the preliminary experiment.

3.2.2 Results

3.2.2.1 Screen the mimotope of the SMC1A FS peptide

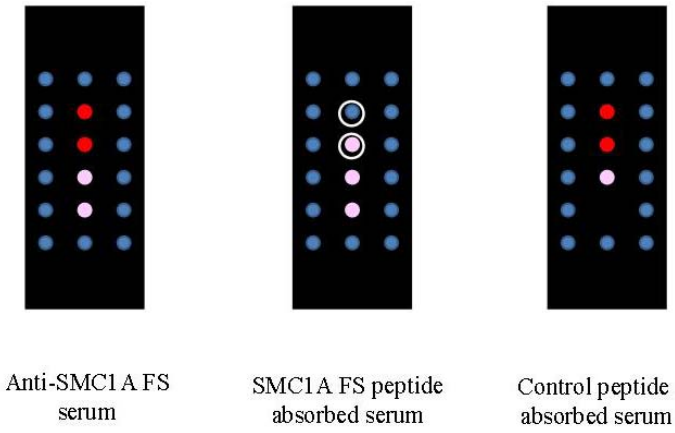


Figure 3. 1 Strategy for mimotope screen. Serum samples with different anti-SMC1A FS peptide activity were applied on the 10K array. The random peptides (circled) that had low reactivity to SMC1A FS peptide absorbed sample and high reactivity to non-absorbed sample and control peptide absorbed sample were the mimotope candidates.

We used CIM 10K random peptide array for the SMC1A FS peptide mimotope screen. The anti-human SMC1A FS peptide serum samples with different anti-SMC1A FS peptide activity were applied on the array. The random peptides that had decreasing reactivity corresponded to the anti-serum samples with decreased antibody activity were the mimotope candidates (Figure 3.1). The rabbit anti-serum that recognized human SMC1A FS peptide (the FS peptide alone with 17 aa) was prepared by the Global Peptide Service LLC. The anti-serum was elicited by immunizing the New Zealand white rabbit of the KLH conjugated human SMC1A FS peptide. This anti serum contained antibody activity against both SMC1A FS peptide and the KLH protein. The samples with decreased anti-SMC1A FS activity were prepared by a step wise specific

absorption with the plates coated by SMC1A FS peptide. The 11 step absorption and 20 step absorption anti-serum were prepared. Their specific anti-SMC1A FS peptide activities were confirmed by ELISA analysis as well as the anti-serum samples that were absorbed by a negative control peptide: AVLLMCQLYQPWMCKEYYRLL (Figure 3.2). The non-absorbed sample, 11 step specific absorbed sample, 20 step specific absorbed sample and 20 step negative absorbed samples were applied on the 10K random peptide array. 50 peptides were selected as the mimotope based on the t-test analysis of the results of different arrays (Figure 3.3).

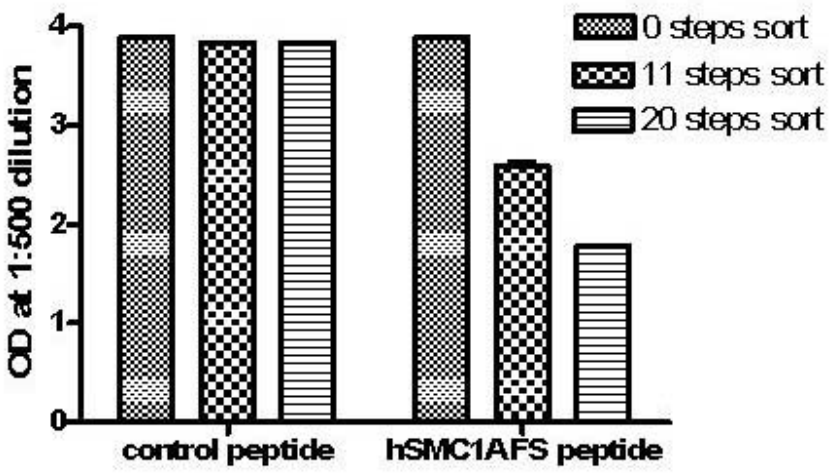


Figure 3. 2 ELISA verification of absorbed anti-serum samples. The antibody against human SMC1A FS peptide in the rabbit anti-serum were specifically absorbed away by the plates coated with SMC1A FS peptide. The anti-serum was also treated with a negative control peptide: AVLLMCQLYQPWMCKEYYRLL

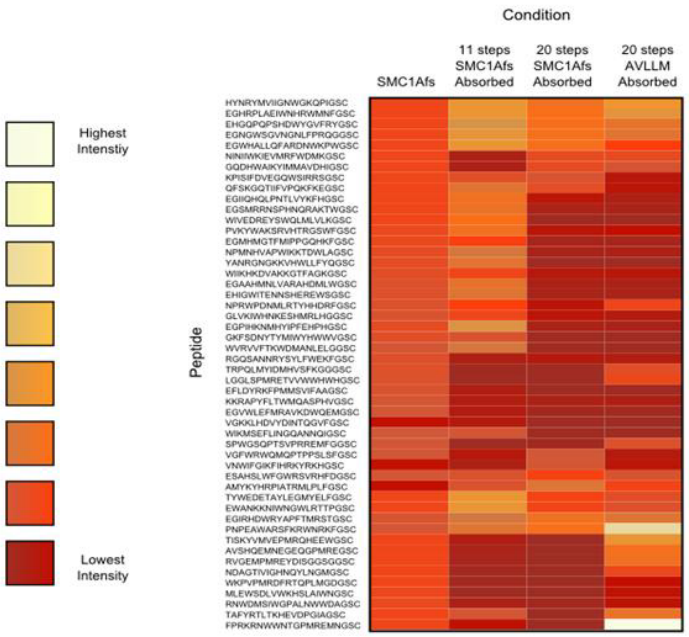


Figure 3. 3 Heat map of intensities exhibited by peptides specific for anti-SMC1A FS antibodies. Four different types of sera were applied to the random peptide microarray: 1. Non-absorbed serum, 2. 11 steps SMC1A FS peptide absorbed, 3. 20 steps SMC1A FS peptide absorbed, 4. 20 steps negative control peptide absorbed. The experiment and analysis were performed by Kurt Whittemore, PhD student at CIM

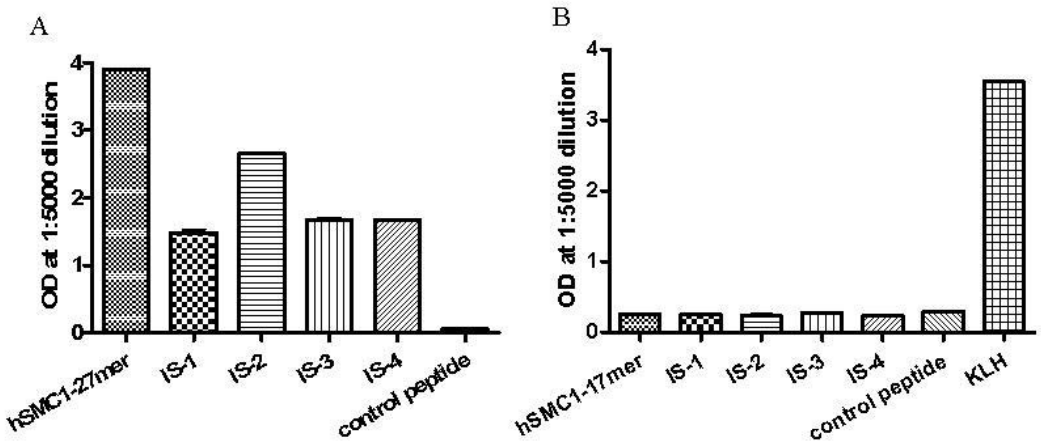


Figure 3. 4 ELISA analysis of the specificity of the mimotope candidates. A. ELISA analysis of the reactivity of the mimotopes with the rabbit anti-hSMC1A FS anti-serum. B. ELISA analysis of the reactivity of the mimotopes with the mouse anti-KLH anti-serum

3.2.2.2 Validation of the mimotopes

We developed a scoring system to evaluate the specificity of these candidates. Each peptide was scored by the following formula: **(max_signal - 20 steps specific_absorbed_signal)/max_signal + (20 step negative_absorbed signal) / max _signal.** The high scoring peptide yield a low signal with 20 steps specific absorbed serum, and a high signal with the 20 steps negative absorbed serum. We chose 4 peptides with the highest score to synthesize and further validate. These peptides were named IS-1 to IS-4 (the array experiment and analysis were performed by Kurt Whittemore, PhD student at CIM). The ELISA analysis of these 4 mimotopes against the rabbit anti-serum demonstrated that these 4 mimotopes could be recognized by the rabbit anti-serum (Fig 3.4 A). To exclude the possibility of the mimotope reactivity to the KLH antibody, I also applied mouse anti-KLH polyclonal anti-serum to these mimotopes. There was no reactivity of these mimotopes to the anti-KLH antibody (Figure 3.4 B). This demonstrated that these 4 mimotopes were specific to the human SMC1A FS peptide. To further validate the specificity of the 4 mimotopes, I synthesized these mimotopes on the Tentagel beads and affinity absorbed the specific antibodies to each peptide from the total IgG of the rabbit anti-serum. Each affinity absorbed antibody could specifically recognize the SMC1A FS peptide by the ELISA analysis (Figure3.5). In the cross reaction analysis, the antibody purified by IS4 mimotope did not have cross reaction to the other 3 mimotopes, while all the other three peptides have cross reaction to each other (Figure3.6). This indicates

the IS4 mimotope represented a different epitope of the human SMC1A FS peptide compared to the other three mimotopes.

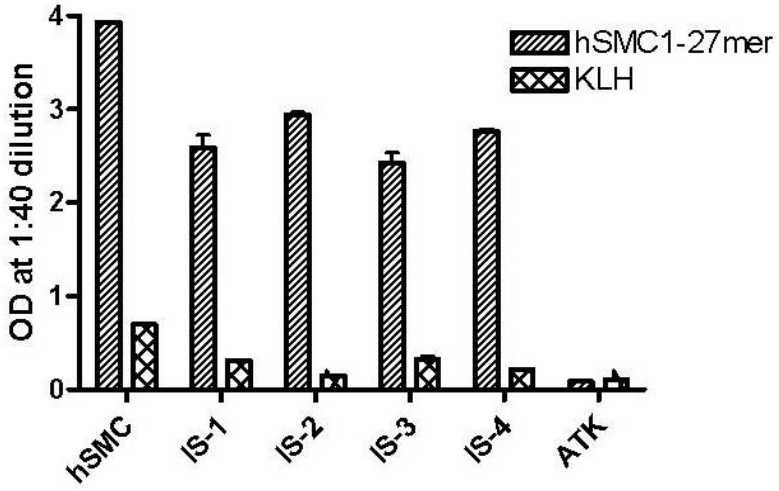


Figure 3. 5 ELISA analysis of antibody that were affinity purified by different peptide against hSMC1A-27mer and KLH protein. ATK is the negative control peptide with the sequence: ATKAAIPGPNTVPRAP

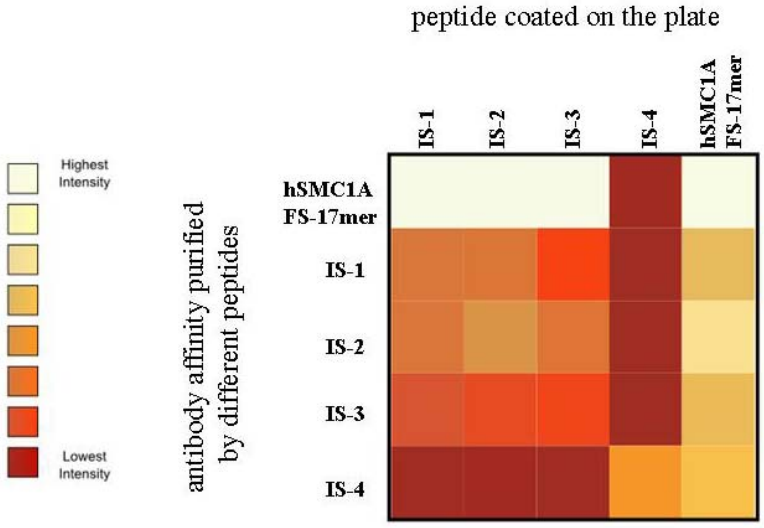


Figure 3. 6 Cross reaction of antibodies that were affinity purified by different peptides

3.2.2.3 Screen cancer patient serum with the mimotopes

To test the concept that mimotopes can be used for FS antibody detection, we printed these 4 mimotopes and the human SMC1A FS peptide on the array and screened the clinical cancer patient serum samples including breast cancer, lung cancer and pancreas cancer, as well as pancreatitis patient and health people (The array experiments performed by Peptide Array Core at CIM). To calculate the positive rate of each cancer type of each peptide, I used a cut-off value by calculating the average intensity plus two fold standard deviation of all healthy people samples. The results were summarized in table 3.1. When we use the original SMC1A FS 17mer peptide, the positive activity rate is almost zero in all cancer patient samples. While using the 4 selected mimotope peptides as a panel, the positive rate increased to 53.8%, 56.5% and 12.5% in breast cancer patient, pancreas cancer patient and lung cancer patient, respectively. The "false positive" rate in healthy people is 10% by using the four peptides as a panel. Interesting, the pancreatitis patients also has high positive rate of 40% by using the 4 peptides panel (Table 3.1).

Table 3. 1 Screen cancer patient's sera. The mimotopes and the human SMC1A FS 27mer peptides were printed on the slides with other peptides that were irrelevant to this topic. The serum of different patients and healthy people were run on the slides. The raw signal intensities of four mimotopes and SMC1A FS 27merwere analyzed. The positive cut-off value is the average intensity plus two fold standard deviation of all healthy people samples.

Case number/Positive (%)	Breast Cancer(n=52)	Pancreas Cancer(n=46)	Pancreatitis (n=20)	Lung Cancer(n=40)	Healthy (n=80)
hSMC 27mer	0/0.0	0/0.0	1/5.0	0/0.0	1/1.3
IS 1	17/32.7	22/47.8	5/25.0	4/10	4/5.0
IS 2	21/40.4	10/21.7	5/25.0	1/2.5	2/2.5
IS-3	17/32.7	1/0.0	1/5.0	0/0.0	2/2.5
IS-4	5/9.6	8/17.4	1/5.0	0/0.0	3/3.8
IS panel	29/53.8	26/56.5	8/40.0	5/12.5	8/10

To further investigation the possible reason of the performance of the mimotopes, I analyzed the possible MHC-II epitopes of the human SMC1A FS peptide and the four mimotopes. I choose the five highest frequency MHC-II alleles in North America from the NCBI bdMHC: DQB1*03:01, DQA1*03:01, DQB1*03:02, DQA1*05:01, DPB1*04:02. I then predicted the binding epitopes of each peptide with the IEDB MHC-II binding predictions. The table 3.2 lists the top 5 peptides with the highest binding affinity to the 5 selected MHC-II alleles. A lower percentile rank indicates higher affinity of the peptide. The binding affinity showed that the human SMC1A FS peptide has lower affinity MHC-II epitopes compared to IS1, IS3 and IS4 in the 5 selected MHC-II alleles. This corresponds to the result of the cancer patient sera screen with the peptide array (Table 3.1). The affinity of the predicted epitopes of IS2 was lower

than human SMC1A FS peptide; however the IS2 performed better than the SMC1A FS peptide in the cancer patient screen. This indicates there are other factors that also could affect the peptide performance, such as the orientation of the peptide on the surface.

I also analyzed the MHC-I epitope of the SMC1A FS peptide and the mimotopes to check whether the mimotopes also have better MHC-I epitopes than the SMC1A FS and have potential to be the vaccine candidates. HLA-A*02:01 is the most frequent MHC-I allele in the US. The HLA-A*02:01 epitope prediction showed that all of four mimotopes have better epitopes than SMC1A FS peptide. Further analysis is necessary to verify the predicted MHC-I and MHC-II epitopes of mimotopes are the mimo-epitopes to the SMC1A FS peptide.

Table 3. 2 MHC II epitope prediction of human SMC1A FS peptide and mimotopes. Epitope prediction was performed with IEDB MHC-II binding predictions and analyzed in 5 highest frequency MHC II allele in North America: DQB1*03:01, DQA1*03:01, DQB1*03:02, DQA1*05:01, DPB1*04:02. The lower percentile rank indicates the higher predicted affinity.

Human SMC1A 27mer: TAIIGPNGSGCCGIYCHEEPQREDSSI		
Allele	Sequence	Percentile Rank
HLA-DQA1*05:01/DQB1*03:01	TAIIGPNGSGCCGIY	30.47
HLA-DQA1*05:01/DQB1*03:01	AIIGPNGSGCCGIYC	31.75
HLA-DQA1*05:01/DQB1*03:01	IIGPNGSGCCGIYCH	35.6
HLA-DQA1*05:01/DQB1*02:01	SGCCGIYCHEEPQRE	39.48
HLA-DQA1*05:01/DQB1*02:01	GCCGIYCHEEPQRED	39.56

IS1: TISKYVMVEPMRQHEEWGSC		
Allele	Sequence	Percentile Rank
HLA-DPA1*03:01/DPB1*04:02	TISKYVMVEPMRQHE	10.38
HLA-DQA1*03:01/DQB1*03:02	TISKYVMVEPMRQHE	14.53
HLA-DQA1*03:01/DQB1*03:02	SKYVMVEPMRQHEEW	17.51
HLA-DQA1*03:01/DQB1*03:02	ISKYVMVEPMRQHEE	17.61
HLA-DPA1*03:01/DPB1*04:02	ISKYVMVEPMRQHEE	18.91

IS2:AVSHQEMNEGEQGPMREGSC		
Allele	Sequence	Percentile Rank
HLA-DQA1*05:01/DQB1*03:01	EMNEGEQGPMREGSC	58.44
HLA-DQA1*05:01/DQB1*03:01	QEMNEGEQGPMREGS	58.78
HLA-DQA1*05:01/DQB1*02:01	AVSHQEMNEGEQGPM	59
HLA-DQA1*05:01/DQB1*03:01	HQEMNEGEQGPMREG	59.42
HLA-DQA1*05:01/DQB1*03:01	SHQEMNEGEQGPMRE	59.94

IS3:RVGEMPMREYDISGGSGGSC		
Allele	Sequence	Percentile Rank
HLA-DQA1*05:01/DQB1*03:01	PMREYDISGGSGGSC	10.93
HLA-DQA1*05:01/DQB1*03:01	MPMREYDISGGSGGS	19.32
HLA-DQA1*05:01/DQB1*03:01	EMPMREYDISGGSGG	45.84
HLA-DQA1*05:01/DQB1*02:01	RVGEMPMREYDISGG	57.65
HLA-DQA1*05:01/DQB1*02:01	VGEMPMREYDISGGS	61.93

IS4: TAFYRTLTKHEVDPGIAGSC		
Allele	Sequence	Percentile Rank
HLA-DQA1*05:01/DQB1*03:01	TLTKHEVDPGIAGSC	16.64
HLA-DPA1*03:01/DPB1*04:02	TAFYRTLTKHEVDPG	33.76
HLA-DQA1*03:01/DQB1*03:02	RTLTKHEVDPGIAGS	34.86
HLA-DQA1*03:01/DQB1*03:02	TLTKHEVDPGIAGSC	36.59
HLA-DQA1*05:01/DQB1*03:01	RTLTKHEVDPGIAGS	37.1

Table 3. 3 HLA-A*02:01 epitope prediction of human SMC1A FS peptide and mimotopes. Epitope prediction was performed with IEDB MHC-I binding predictions with artificial neural network. Lower IC50 value indicates better binder.

Human SMC1A 27mer: TAIIGPNGSGCCGIYCHEEPQREDSSI	
Sequence	IC50 (nM)
GIYCHEEPQ	20045.2
GSGCCGIYC	22738.55
IIGPNGSGC	22887.76
TAIIGPNGS	23965.2
CCGIYCHEE	23991.01

IS1: TISKYVMVEPMRQHEEWGSC	
Sequence	IC50 (nM)
YVMVEPMRQ	15406.97
RQHEEWGSC	20721.32
TISKYVMVEP	20915.49
SKYVMVEPM	21453.88
YVMVEPMRQH	22713.59

IS2:AVSHQEMNEGEQGPMREGSC	
Sequence	IC50 (nM)
EMNEGEQGPM	16705.92
MNEGEQGPM	23743.36
EQGPMREGS	24226.43
VSHQEMNEG	24317.42
QEMNEGEQG	24498.46

IS3:RVGEMPMREYDISGGSGGSC	
Sequence	IC50 (nM)
EMPMREYDI	14449.96
GEMPMREYDI	17781.54
DISGGSGGS	23340.91
REYDISGGS	23704.35
RVGEMPMRE	23758.65

IS4: TAFYRTLTKHEVDPGIAGSC	
Sequence	IC50 (nM)
LTKHEVDPGI	10176.12
FYRTLTKHEV	19438.72
YRTLTKHEV	22151.74
TKHEVDPGI	22362.21
EVDPGIAGS	23159.04

3.2.3 Discussion

Through this preliminary experiment, I demonstrated that the specific antibody activity to the SMC1A FS peptide has potential to be a biomarker for the diagnosis of breast cancer and pancreas cancer. By using 4 mimotope peptide panel of the SMC1A FS peptide, I detected the antibody activity to the SMC1A FS peptide from 50% to 60% of the breast cancer patients and pancreas cancer patients. Only 10% normal healthy people showed the positive antibody activity to the SMC1A FS peptide. This positive rate of the antibody against SMC1A FS peptide is about two fold higher than currently reported rate of single autoantibody detection in cancer patients, which is only 20% to 30% [188]. Since the SMC1A FS transcript was detected in all the different types of tumor samples that we have screened so far, the antibody against the SMC1A FS peptide may be

a general cancer biomarker. It is interesting that the specific antibody against the SMC1A FS peptide was also detected in pancreatitis patients with 40% positive rate by using the panel of the 4 mimotope peptides. Pancreatitis is highly associated with pancreas cancer. This indicates antibody against the SMC1A FS peptide might be used for early pancreatic cancer diagnosis. To confirm the antibody against the SMC1A FS as an early cancer biomarker, further investigation is needed to be performed on more samples with detailed clinical information.

The preliminary data also demonstrated the unique advantage of the specific antibody detection strategy by using the mimotope peptides. Different cancer patients have different antibody activity to the 4 selected mimotopes. This indicates the diversity of the antibodies against the SMC1A FS peptide in the population. Each patient has a unique antibody profile to the SMC1A FS peptide. The sensitivity of the antibody detection with the human SMC1A FS peptide itself was very low. The positive rates of the antibody directly against the human SMC1A FS peptide in different cancers were almost 0. While the positive rate of antibody detection of the panel of the 4 selected mimotope peptides were from 12.5% to 56.5%. Each individual mimotope was more reactive to the patient serum than the original SMC1A FS peptide. This may, however, be due to technical problems with binding the SMC1A FS peptide to the surface. The MHC-II binding affinity prediction showed that the MHC-II epitopes of mimotopes IS1, IS2 and IS4 had higher affinity than the predicted MHC-II

epitope of SMC1A FS peptide in the 5 selected MHC-II alleles. This indicated that these mimotopes could have higher affinity to the antibodies generated by the human SMC1A FS peptide with the 5 selected MHC-II alleles. This may be one of the reasons that these mimotopes were more sensitive in detecting the antibody against the SMC1A FS peptide. Another possibility is that the mimotope, such as IS3, has higher sensitivity because the FS peptide orientation on the surface is not controlled and could not present the epitope correctly, while the mimotopes are selected to function on the surface. Therefore, some mimotopes with lower predicted affinity still could perform better than the FS peptide. In summary, this result demonstrates that the mimotope not only can efficiently present all possible epitopes, but also can present higher affinity epitopes. Using the mimotopes for the specific antigen is an advanced strategy for increasing the sensitivity of antibody detection. This strategy is general and could also improve the detection of the autoantibody that is against self-antigens, which usually contain multiple epitopes. The low activity of SMC1A FS antibodies in lung cancer patients may reflect the low immunogenicity of lung tumors, which has been reported by various studies on lung cancer. This also may be caused by the different epitopes present on lung tumor cells. The sensitivity of the detection in lung cancer patients could be improved by including more mimic peptides, which could present different epitopes of the SMC1A FS peptide.

The successful detection of the antibody activity against SMC1A FS peptide in cancer patients also suggests the importance of the antibody activity to

cancer related FS peptides, and therefore their potential as biomarkers for cancer diagnosis. As I described above, the FS transcripts were frequently detected in different cancers. Further investigation of antibodies against other cancer related FS peptides will identify more effective biomarkers for early cancer diagnosis. With the panel of antibodies against different FS peptides, it should improve the sensitivity and specificity for early cancer diagnosis.

Detection of the antibody activity against SMC1A FS peptide in cancer patients also provides the evidence of the expression of the SMC1A FS peptide in these tumor cells. This suggests the SMC1A FS peptide could be a valuable cancer vaccine candidate. The investigation of the FS-antibody in cancer patients not only could develop this as a unique cancer biomarker, but also could evaluate the potential of the related FS peptides as cancer vaccine candidates. In addition, as discused above, the mimotopes may serve as better antigens than the FS peptide itself.

The successful detection of the antibody against the SMC1A FS peptide with a set of mimotopes also directly indicates the biological significance of the immunosignature technology for disease diagnosis. The technology of immunosignature was developed by our lab to diagnose different diseases by profiling antibody activity with random peptides arrays. We clearly demonstrated that the immunosignature could differentiate cancers patients from the healthy people without knowledge of the specific disease related targets of the antibodies. I applied the same platform to screen the mimotopes of the SMC1A FS peptide. 4

mimotope peptides of the list were confirmed that had high antibody activity in cancers patients. The detected antibody activities were specific to MC1A FS peptide. Therefore, it demonstrates that the antibodies against the SMC1A FS peptide contribute to the antibody profiling of cancer patients compared to healthy individuals by immunosignature analysis.

3.3 Circulating FS transcript as cancer biomarker

3.3.1 Introduction

Circulating nucleic acids (cNAs) were first detected by Mandel and Metais [189]. The cNAs were not attractive to scientists for diagnosis until the detection of mutated RAS gene in the blood of cancer patients in 1994 [190]. Since then, the various cNAs (such as DNA, mRNA and more recently micro RNA) were detected in patients and healthy populations. These cNAs are released by tumor and normal cells and often diseased cells, and protected from nucleases by proteins or micro vesicles [191-193]. Disease related cNAs became popular as a biomarker for diagnostics, especially for early cancer diagnosis. A long list of cNAs have been reported as cancer biomarkers, however there is no single biomarker that is widely used in the clinic. The main problem of the cNAs biomarker development is quantification. Most cancer related circulating mRNA and micro RNA needs to be quantified for the diagnosis, while the reality is there is no reliable reference for the circulating cNAs quantification. More, the cNA quantification is highly sensitive to the sample process and there is no standard assay for cNAs quantification. All of these make the current cNAs

biomarkers that were reported in the literature not widely used for clinical applications. However, many companies are pushing this diagnostic strategy. Digital PCR has created new potential in this area.

As I described above, we identified a list of the FS transcripts from the bioinformatics analysis and validated by RT-PCR of the tumor cDNAs. One type of FS transcripts is caused by insertion or deletion of a nucleic acid fragment. Those FS transcripts were frequently generated in different cancers, such as the SMC1A FS transcript. Both WT and FS transcripts of the SMC1A could be amplified by a set of flanking primer from tumor cDNA and separated by the gel electrophoresis. The Q-PCR analysis of the SMC1A FS transcript in human primary breast tumor and normal mammary glands demonstrated that the SMC1A FS transcript was highly expressed in breast tumor cells. Here I investigated a hypothesis that this highly expressed SMC1A FS transcript could be detected in the blood, and it could be a unique cancer biomarker. I discovered that the SMC1A FS transcript also existed in the blood of tumor bearing mice. I also developed a unique method to correlate the level of both SMC1A FS and WT transcripts with the tumor development by the regular RT-PCR and gel electrophoresis.

3.3.2 Result

3.3.2.1 Discover the SMC1A WT and FS transcript in tumor bearing mice and healthy mice

The experimental design for SMC1A WT and FS transcript detection was straight forward. The total circulating RNA was purified from the mouse plasma by Trizol-ethanol precipitation. This protocol will extract both free and protein bound RNA. The total circulating RNA was reverse transcribed into cDNA and then used for regular PCR analysis with the flanking primers that can amplify both WT and FS transcript of the SMC1A (Figure 3.7).

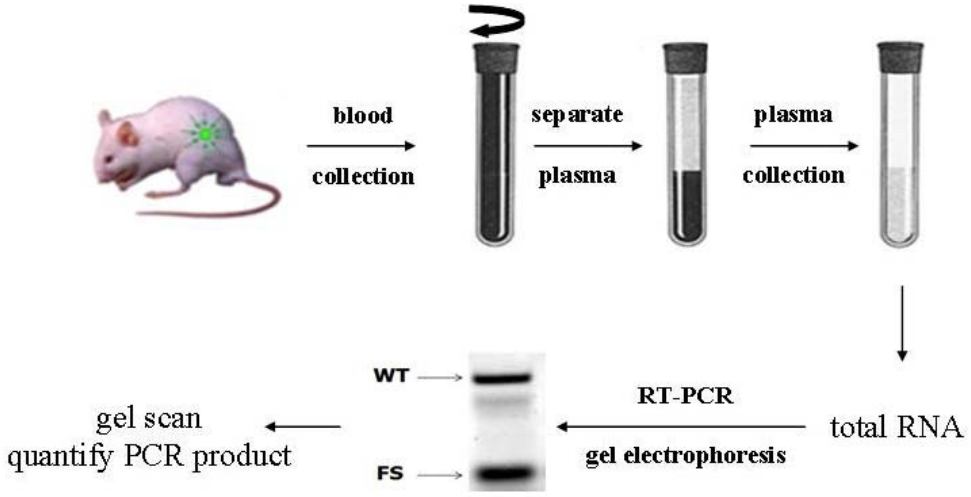


Figure 3. 7 Process of the analysis of the circulating SMC1A FS and WT transcript. Mouse blood was collected by cardiac puncture and centrifuged for plasma collection. Total circulating RNA was extracted from the plasma by Trizol-ethanol precipitation followed by RT-PCR with flanking primers for amplification of both SMC1A FS and WT transcripts. The PCR products were analyzed by gel electrophoresis and gel scan to quantify the amplified SMC1A FS and WT transcripts.

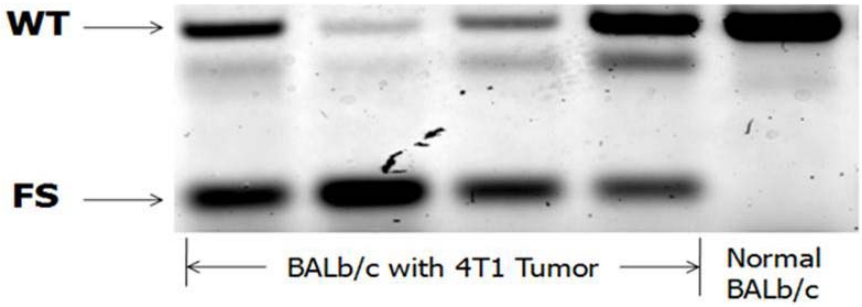


Figure 3. 8 Gel electrophoresis of the RT-PCR products of the circulating RNA from the blood. 4 end-point 4T1 tumor bearing mice and 1 healthy mouse were included in the assay

Tumor bearing mice were established by inoculating $5x10^3$ 4T1 tumor cells into the mouse mammary glands. The mice were euthanized and the plasmas were collected when tumor volume increased to 2 cm^3 according to the approved protocol. 4 tumor bearing mice and 1 healthy mouse were included in the first trial. The PCR products were analyzed by regular gel electrophoresis. Both WT and FS transcript of the SMC1A were detected in the plasma from tumor bearing mice and only the WT transcript of the SMC1A was detected in the healthy mouse (Figure 3.8). It is interesting that the FS transcript of the SMC1A was predominant relative to the WT transcript in three of four PCR products from plasma of tumor bearing mice. This discovery led me to further investigate the possibility of using this pattern of the SMC1A FS and WT transcript as a cancer biomarker. Importantly, the ratio of the WT and FS RNAs could produce an internal control.

3.3.2.2 Correlation between tumor size and ratio of the WT and FS transcript by regular RT-PCR of the circulating RNA

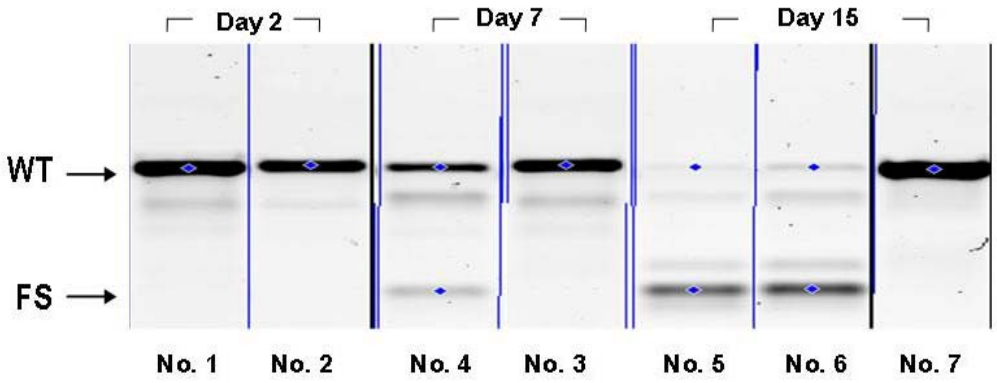


Figure 3. 9 Analysis of the circulating SMC1A FS and WT transcripts with 4T1-BALB/c mouse breast tumor model. $5x10^3$ 4T1 cell were inoculated into mouse mammary gland. The circulating RNA was extracted from mice at 2 days, 7 days and 15 days after tumor inoculation as indicated at the top of the fig. All of the total RNA samples were processed the same time for RT-PCR and gel electrophoresis analysis. The gel was scanned and PCR products of the WT and FS transcripts were labeled and quantified.

To investigate the correlation between tumor status and the detection of WT and FS transcript by regular RT-PCR, I set up a time sensitive experiment. The plasma samples were collected at different time points after 4T1 cell inoculation. To exclude the possibility that circulating FS transcripts were directly released during the process of inoculating the tumor cells, plasma samples were collected 2 days after 4T1 tumor cell inoculation. 7 days after tumor inoculation was another time point to investigate the early detection since most mice did not develop palpable tumor by that time. At 15 days after tumor inoculation most mice develop palpable 4T1 tumors with variable tumor size. The cDNA from the total circulating RNA was prepared at the time of the plasma collections and the

PCR analysis performed at the same time (Figure 3.9). There was no detectably circulation of FS transcripts of SMC1A in the plasma samples collected 2 days after tumor inoculation. The FS transcripts started to be detected with plasma collected 7 days after tumor inoculation and the intensity of the PCR product of the FS transcript trended to increase when the tumor further developed, while the WT transcript PCR products trended to decrease. To quantify the change of the PCR amplified WT and FS transcript and investigate the correlation between this change and the tumor development, I calculated the ratio of the PCR amplified FS verse WT transcripts by scanning the electrophoresis gel and quantifying the intensity of both bands (Figure 3.10 A). The ratio increased with the time of tumor development. I also discovered that the tumor size was positively correlated with the ratio of the PCR amplified FS transcripts verse the WT transcripts (Figure 3.10 B).

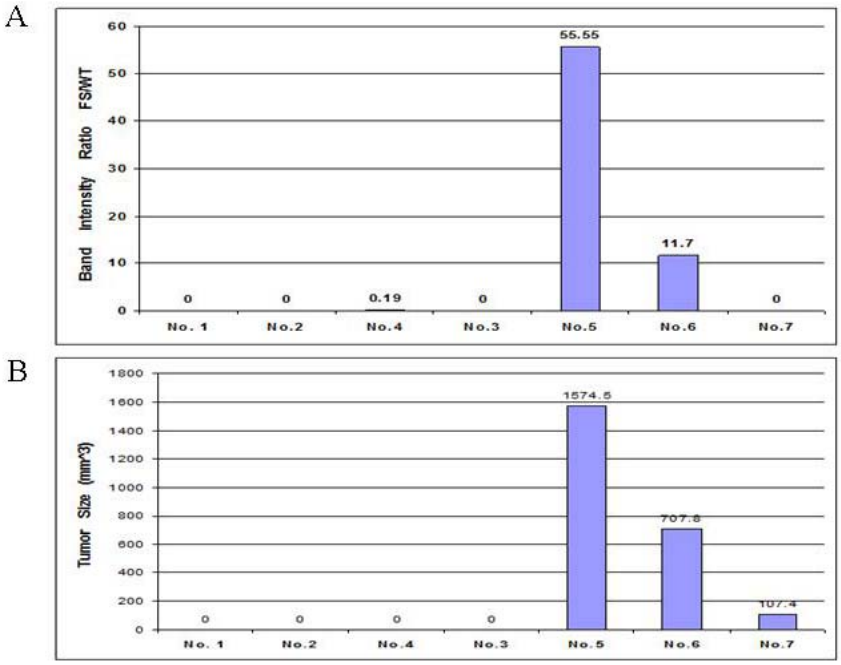


Figure 3. 10 Correlation between tumor development and the PCR products ratio of the FS and WT transcripts. A. the ratio of the quantified PCR products of the FS and WT transcripts of each plasma samples. B. palpable 4T1 tumor size of each mouse by the time of the blood collection.

3.3.3 Discussion

With the experiments that were described above, I demonstrated that the ratio of the PCR amplified SMC1A FS transcript verse the WT transcript could be used as a biomarker for early cancer diagnosis as well as tumor development. I showed that both the circulating SMC1A WT and FS transcripts could be detected from the tumor bearing mice, while only the WT transcript could be detected in healthy mice. There is no detectable circulating FS transcript in plasma collected 2 days after tumor cell inoculation. This indicated that the detected FS transcripts in later stages were not directly released by the inoculated tumor cells. They

should be specifically released by developing tumors. The circulating FS transcript could be detected as early as 7 days after tumor cell inoculation at which time there is no palpable tumor. I also demonstrated that the ratio of the PCR amplified FS transcript verse the WT transcript was positively correlated with tumor development. The absence of detectable FS transcript in two tumor bearing mice may be caused by the procedure process, such as contamination of the RNA from blood cell by erythrocytolysis. This also may simply be caused by the small number of tumor cells in these two mice. Further investigation of protocol optimization is necessary.

Comparing other currently studied cNAs biomarkers, this biomarker represents advantages by simplifying the analysis and keeping the sensitivity. Unlike other cNAs analysis, this unique biomarker analysis does not need the quantitative PCR analysis, which is limited by the reference and also sensitive to the sample process. In this unique biomarker analysis, the circulating WT transcript acts as the internal reference for the increase of the circulating FS transcript and this reference factor is included in the calculation of the ratio of the PCR products. The analysis of this biomarker could be accomplished by the regular RT-PCR and gel electrophoresis, which is more simple and robust.

There are some unique characteristics of this biomarker that improve the sensitivity and specificity of this biomarker analysis. First, the signal of this biomarker is amplified in vivo by natural characteristics. Studies showed that the smaller fragments of the cNAs were more frequent and stable in the blood. This

suggested that the target fragment of the SMC1A FS transcript tends to be more enriched in the blood than the target fragment of the WT transcript, which is 302bp longer. Therefore, the target FS fragments that are released by the tumor cells should be more efficiently accumulated in the blood than the target WT fragment. Consequently, this increases the sensitivity of the detection. This is in contrast to most approaches to monitoring cancer cNAs in the blood which focus on mutations. Because of the difficulties in PCR monitoring of mutations, this approach usually requires using digital PCR. Secondly, the sensitivity of this biomarker in vitro is improved by the unique setting of the assay. The PCR amplifications of the FS and WT fragments in the same reaction compete for the same primer set. The shorter FS fragment is more efficient to be amplified than the longer WT fragment. Therefore, the signal of this biomarker is further amplified. This may explain why there is less WT fragment as more FS is present in Figure 3.9.

The proof of concept study of this biomarker by analysis of the circulating SMC1A WT and FS transcripts suggests wide applications of this biomarker and others like it. First, this indicates that all of the cancer related splicing variants could be candidates for this biomarker analysis. A list of the tissue specific and cancer specific splicing variants have been identified [194] (Lee and Johnston, in preparation). We could develop the general and specific cancer biomarkers by analyzing the splicing profile of these biomarkers. Second, this class of biomarkers could not only be used for early cancer diagnosis, but also could be

used for the cancer treatment monitoring, such as tumor metastasis and recurrence. The studies of the circulating cNAs showed that the level of the cancer related cNAs significantly decreased after the treatment, such as surgery [195]. The sensitivity of this biomarker to the cancer related transcripts suggests its important value for the treatment, evaluation and monitoring.

In summary, this proof of concept study suggests that this biomarker analysis has an important value for the early cancer diagnosis and treatment monitoring. Further investigation should focus on the optimization of the biomarker analysis, such as circulating RNA isolation and PCR analysis. We also should evaluate this biomarker in more animal tumor model and clinical samples and screen more candidate targets.

3.4 Materials and Methods

3.4.1 Rabbit SMC1fs Serum

Rabbit anti-SMC1A FS peptide serum was produced for me by Global Peptide Service LLC. The New Zealand white rabbit was immunized with KLH conjugated human SMC1A FS peptide with a sequence of CCGIYCHEEPQREDSSI.

3.4.2 Antibody absorption

Specific antibodies were absorbed from the rabbit anti-SMC1A FS serum by applying the serum to the hSMC1Afs 17mer peptide coated plate. The amino acid sequence of this hSMC1Afs- 17mer peptide was

CCGIYCHEEPQREDSSI. In some experiments, the hSMC1Afs-27mer peptide was used which contains 10 amino acids of wild type sequence upstream of the hSMC1Afs-17mer for better epitope representation. The amino acid sequence of the hSMC1Afs-27mer is TAIIGPNGSGCCGIYCHEEPQREDSSI . The rabbit serum was diluted 1:250 with 3% BSA in PBST and incubated with the hSMCfs peptide coated plate at 37°C for 1 hour. The unbound antibody in the supernatant was then removed and applied to another peptide coated well to remove more antibody specific for the peptide. This process was repeated up to 20 times, and this serum was then later applied to the peptide microarray with a dilution of 1:500. This same method was used to produce negative control, antibody absorbed serum using the negative control AVLLM peptide with a sequence of AVLLMCQLYQPWMCKEYYRLL.

3.4.3 Random peptide array printing

10,000 peptides made up of a randomly generated sequence were chemically synthesized. These peptides contain 17 random amino acids and 3 amino acids on the C terminus with the sequence of GSC. The C terminal cysteine binds to a sulfo-SMCC coated aminosilane glass slide. The solutions of different random peptides were spotted onto the glass slide using a Nanoprint 60 instrument.

3.4.4 Customized peptide array

Peptides were synthesized by Sigma and printed with another 144 peptides by AMI.

3.4.5 Application of serum to random peptide array

Rabbit serum was applied to the random peptide microarray using a Tecan HS 4800 Pro microarray hybridization station. Slides were first washed for 30s with TBST, and then blocked with a blocking buffer consisting of BSA, mercaptohexanol, Tween 20, and PBS for 1 hr at 23 C. Duplicate samples of serum were diluted 1:500 in an incubation buffer consisting of BSA, Tween 20, and PBS and incubated with the slide for 1 hr at 37 C. The slide was then washed, and 5 nM of goat anti-rabbit AlexaFluor 647 dye was applied for 1 hr at 37 C. The slide was then washed and dried for 5 minutes.

3.4.6 Scanning and analysis of array

The slides were scanned with an Agilent Technologies DNA Microarray Scanner with Surescan High-Resolution Technology instrument and analyzed with GenePix Pro 6.0 software to determine the fluorescence intensity of each spot. GeneSpring GX 7.3, Microsoft Excel, simple custom Java code, and GraphPad Prism 4 were then used to perform further analysis of this data.

3.4.7 Antibody purification

Specific antibodies were purified from serum by flowing serum through a column filled with TentaGel beads with synthesized peptides on their

surface such as the SMC1Afs peptide, selected random peptides, or an irrelevant ATKAA peptide as a negative control with a sequence of ATKAAIPGPNTVPRAP. The total IgG of the rabbit anti-hSMC1A FS serum was purified using Pierce Protein A/G Agarose beads with the protocol of the manufacturer. 1 ml of the TentaGel beads were mixed with 3ml of the purified total IgG, and this solution was incubated for 1 hour at room temperature. The column was then washed with 10ml PBST. The specific IgG was eluted with 4 fractions of 1ml IgG Elution Buffer from Pierce and each fraction was neutralized with 100 uL 1M Tris. All of the eluted fractions were measured at an absorbance of 280nm. The two fractions with the highest absorbance were combined and used for further analysis at a 1 to 40 dilution in 3% BSA in PBST.

3.4.8 Human serum samples

Center for Innovations in Medicine, Biodesign Imstitute, Arizona State University holds IRB 0912004625.

3.4.9 Circulating RNA extraction and RT-PCR

The circulating RNA extraction was modified from the manufacter's protocol for TRIzol. Mix 250ul mouse plasma with 1ml TRIzol and vortex for 30 seconds and let sit at room temperature for 5 minutes. Add 20ul chloroform and shake for 30 seconds. Sit at room temperature for 2 minutes. Then centrifuge the mix 12,000xg for 15 minutes and transfer the aqueous phase to another tube. Mix the aqueous phase with 500ul isopropyl alcohol and incubate at -20°C for 15

minutes. Then centrifuge 12,000xg for 30 minutes at 4°C. Remove the supernatant and wash the pellet with cold 75% ethanol. Centrifuge 8000xg for 10 minutes. Then remove the supernatant and air-dry the pellet. Then dissolve the pellet in 30ul RNase free water.

The RNA concentration was too low to measure with the Nano-drop. Therefore I directly use 8ul for cDNA synthesizing with SuperScript™ First-Stand Synthesis SuperMix (Life Technologies, Carlsbad, CA). The end-point PCR was performed with Gotaq™ PCR kit (Promega Corporation, Madison, WI) and Mastercycler ep gradient S (Eppendorf, Hamburg, Germany).

3.4.10 Gel electrophoresis and quantifing the intensity of the PCR products

The PCR product was analyzed by gel electrophoresis with 2% argrose containing EB. The gel was scanned on the Typhoon. The intensity of each band was quantified by Image Quant 5.2. The ratio of the FS fraction verse the WT fraction was calculated by the intensity.

CHAPTER 4

SMC1A AND TUMORIGENICITY

4.1 Introduction

The cancer related genes, such as tumor suppressor genes and oncogenes, are the traditional topic of cancer research. Dozens of cancer related genes have been characterized [196]. These cancer related genes are involved in each step of tumor development, from tumor initiation to metastasis. Cancer related genes that are triggered by genetic and epigenetic alterations are well studied. More recently, the accumulated evidence shows that the mis-regulated alternative splicing also plays an important role during tumor development by affecting genes that are involved in almost every aspect of tumor progression; such as metabolism, cell cycle control, apoptosis, invasion and metastasis [194, 197]. Alternative splicing increases the diversity of the genome. It is tightly regulated in normal cells by specifically expressing different isoforms with different functions in different stages of the development of different tissues [198]. The tumor cells take advantage of this regulation system of alternative splicing and triggers the splicing isoforms that is not normally expressed in normal adult cells. A major type of mis-regulated splicing in cancer cells is the expression of isoforms that encode proteins with appositive functions that were encoded by isoforms expressed in normal cells [199]. The expression of these mis-regulated splicing isoforms increases the advantage for tumor progression. For example, Bcl-x has two isoforms with different sizes that are generated by alternative splicing at

two splice sites at the 5' end of the exon2 [200]. The longer isoform of Bcl-x has anti-apoptosis function, while the short isoform could induce the apoptosis of the cancer cells. The bias of the splicing to the long isoform of Bcl-x is observed in a variety of types of cancers [201-204]. Some other mis-regulated splicing isoforms have unknown functions but reduce the normal functional isoforms by consuming the pre-mRNA. For example, the caspase-2 gene with full length splicing isoform (caspas-2L) is involved in a variety of cellular processes and acts as a tumor suppressor gene [205, 206]. The mis-regulated splicing isoform of the caspase-2 (caspase-2S) contains an additional exon (exon9). The splicing of exon9 causes a frameshift and generates a PTC in the caspase-2S, which makes it a target of the NMD. The reduction of the expression of the caspase-2 was observed in different tumors and this may be caused by mis-regulated splicing of the caspase-2S and degradation by NMD [207, 208].

During my research on neo prophylactic cancer vaccine candidate discovery, we identified the SMC1A FS alternative splicing by directly splicing exon 1 to exon 4 of the SMC1A pre-mRNA which generates a FS transcript with a PTC in exon4 (Figure 1.5). This SMC1A FS transcript was highly expressed in primary human breast tumors (Figure 1.6). The SMC1A FS transcript was also detected in a variety of types of human tumors, mouse tumors and dog tumors with highly conserved sequences, as I described above (Figure 1.7-1.9).

The WT SMC1A has important functions in different cellular activities. SMC1A was first identified in *Saccharomyces cerevisiae* by studying a mutant

strain with unstable maintenance of minichromosomes [209]. It is an important component of a cohesion complex which is formed by a heterodyne of SMC1A and SMC3 and two non-SMC proteins: Scc1 and Scc3. The primary function of cohesin is in holding sister chromatids together by forming rings that locate along the chromosome during DNA replication in S phase until anaphase. It stabilizes the chromosome. The homozygous deletion of Smc1A in *S.cerevisiae* is lethal and heterozygous deletion causes haploinsufficiency [210, 211].

Recently, cohesin was found to be involved in the regulation of gene expression. In Drosophila, the cohesin homologue is the Nipped-B protein. The Nipped B binding sites in the transcribed regions in the Drosophila genome overlap with RNA polymerase II. The binding of both cohesin and Nipped-B is associated with the expression of genes located in these regions [212, 213]. In mammals, cohesin is co-localized with zinc finger transcription factor CTCF along the mammalian genome. CTCF functions as an enhancer-blocking transcriptional insulator. Studies indicate its function depends on interaction with cohesion [214-216].

SMC1A is also involved in DNA repair mechanisms. The early indication of SMC1A being involved in DNA repair was finding that the SMC1A/SMC3, DNA polymerase E and DNA ligase III are involved in the RC-1 complex, which can recombine homologous DNA and repair both gaps and deletions in those DNA in vitro [217]. Studies in yeast show impaired DNA double stranded break repair through the homologous recombination pathway in SMC1A mutated cells

[218]. It also shows recruitment of SMC1A around DNA double stranded break sites even in the G2 phase of cell cycle division [219]. In mammalian cells, SMC1A is phosphorylated by ataxia telangiectasia mutated (ATM) after ionizing irradiation. Mutation of the phosphorylation site of SMC1A will decrease cell survival only after DNA damage. This suggests a role of SMC1A in DNA repair that is independent of its essential role in cohensin [220].

Since SMC1A has such important functions in different aspects of cellular processes, people suspected that the alteration of SMC1A is related to inherited diseases. The genetic mutations of SMC1A are well characterized with being involved in the genetic disease Cornelia de Lange Syndrome (CdLS). CdLS is a developmental disorder of multiple systems. The typical syndromes of this heterogeneous disease are consistent facial dysmorphia, upper extremity malformations, hirsutism cardiac defects and so on. Multiples gene mutations are identified that cause this disease, such as NIPBL, PDS5B, SMC3 and SMC1A [221-224]. The mutation of the SMC1A is responsible for 3% of CdLS cases. The mutation of the SMC1A is also detected in human colorectal cancers [225]. The mutation of SMC1A hypothetically causes a defect of the cohesin, consequently inducing chromosomal instability, which is a hallmark of colorectal cancers. The alteration of the SMC1A is also correlated with acute myeloid leukemia (AML). Low or absent SMC1A protein expression is detected in 64% AML specimens and the low SMC1A protein expression is significantly correlated with shorter event free and overall survival of the AML patients [226]. There is no significant

correlation between the mRNA level and the protein level of SMC1A in those AML patients. This indicates there are epigenetic alternations of the SMC1A in AML patients. It is also reported that knocking down SMC1A expression by antisense oligonucleotide treatment of the primary human fibroblast cell line could induce both aneuploidy and chromosome aberrations. However, there was no tumorigenicity induced by measuring the anchorage independent growth [227]. So far, there is no direct evidence demonstrating that either decreasing the WT SMC1A expression, or increasing the SMC1A FS expression, or both could increase the tumorigenicity of the normal cells.

Our findings with SMC1A correlate with previous literature reports, and has lead to the hypothesis that **SMC1A is involved in the general tumor development through its mis-splicing. This mis-regulated splicing either decreases the expression of the WT transcript by consuming the pre-mRNA of SMC1A, has the function to increase the tumorigenicity of the normal cells, or both effects.**

To test this hypothesis, I investigated the expression of both WT and FS isoforms of SMC1A in tumor and normal tissues. I also directly tested the tumorigenicity induction by knocking down the expression of the WT isoform in non-tumorigenic cell lines.

4.2 Result

4.2.1 Detection of the alternative splicing of SMC1A in tumor

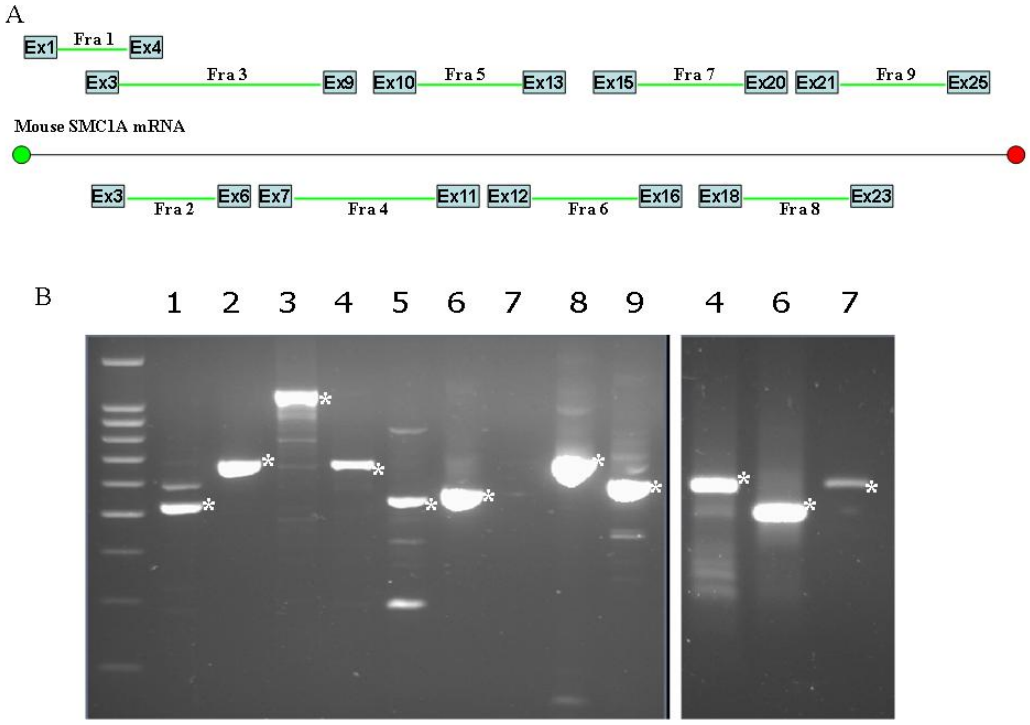


Figure 4. 1 Detection of alternative splicing of the SMC1A. A. Whole mature SMC1A mRNA were divided into 9 overlap fragments. 9 sets of primer were designed for amplifying each fragment. B. RT-PCR of B16F10 cDNA with the 9 sets primers. Starts indicate the PCR products with predicted WT size.

SMC1A FS is detected in a of variety tumors. To test if there are other alternative splicings of the SMC1A in tumors, I analyzed all alternative splicing of the SMC1A in B16F10 cells (mouse melanoma cell line) by RT-PCR with 9 sets of the primers across the whole SMC1A exons (Figure 4.1 A). The whole CDS of SMC1A was divided into 9 overlapping fragments and each fragment was amplified by RT-PCR with a set of primers and analyzed by gel electrophoresis (Figure 4.1 B). However, because of the different primer sets and PCR

conditions, the SMC1A FS transcript could not be efficiently amplified in this experiment. The result demonstrated that there are several alternative splicing transcripts of SMC1A in B16F10 tumor cells with a variety of expression levels. The PCR products with shorter lengths of the fragments 3, 4 and 9 were cloned and analyzed by clonal sequence. A total of 30 clones were sequenced with clear traces. 7 clones were WT sequences, 6 clones were FS deletions, and 17 clones were in frame deletions. A total of 18 different deletions were detected. All of the deletions, except one, were not perfect exon skipped splicing. They were spliced from the middle of the exon to another middle of an exon (Figure 4.2). These results indicated that the splicing of the SMC1A was mis-regulated in the tumor cells. The splicing of the WT isoform may be affected by these mis-regulated alternative splicing mechanisms.

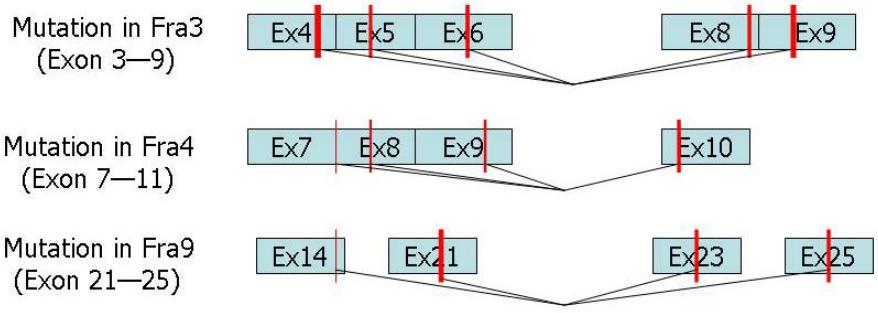


Figure 4. 2 Sequence analysis of the alternative splicing of the SMC1A. The alternative splicing from the 3 fragment RT-PCR were cloned and sequenced. Total 17 alternative splicing were detected by sequencing 30 clones. The red bars indicate the approximate splicing sites of each alternative splicing. The width of the bar indicates the splicing number, wider bar indicate more splicing.

4.2.2 The SMC1A WT is down regulated and the FS is up regulated in pancreatic tumors

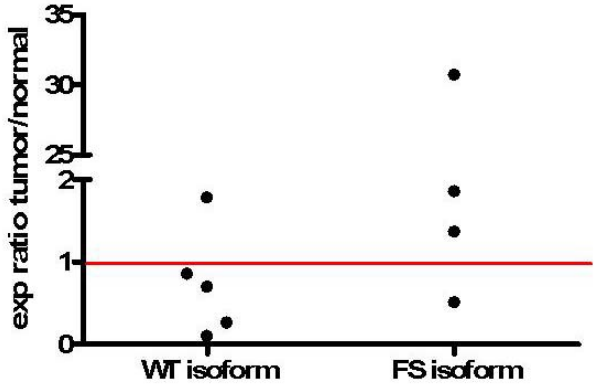


Figure 4. 3 q-PCR analysis of the WT and FS isoforms expression in pancreas tumors and the normal adjacent tissues. The expression of the WT and FS isoform were measured in patient matched pancreas tumor and normal adjacent tissues. The expression ratios of each isoform in tumor verse normal tissue were calculated with the actin as the reference. The ratio below 1 indicates the isoform expression level in tumor is less than normal and vice versa.

The mis-regulated alternative splicing could not only increase the expression of the oncogenic isoforms, but also could decrease the expression of the tumor suppression isoforms. The SMC1A FS splicing is frequently increased in human breast tumors. To test the hypothesis that the increasing SMC1A FS splicing decreases the WT splicing by consuming the pre-mRNA of the SMC1A, I investigated the expression of both WT and FS isoforms in the patient matched pancreas tumor and normal adjacent tissue samples. To accurately compare both relative isoforms, the WT isoform was measured by a primer pair that could amplify a fragment within exon2 and exon3. Both exon2 and exon3 were skipped in the FS isoform. The FS isoform was measured by a primer pair with the 5'

end primer located at the junction of the exon1 and exon4. This primer pair could specifically amplify the FS isoform. A total of 5 pairs of the patient matched samples were analyzed by q-PCR. The expression ratio of the WT isoform in the tumor sample verse the matched normal adjacent tissue was calculated, as well as the expression ratio of the FS isoform (Figure 4.3). The results demonstrated that the WT isoform was frequently down regulated in pancreatic tumors, while the FS isoform was frequently up-regulated in pancreatic tumors.

However, two pairs of patient matched samples have both WT and FS isoforms up-regulated or down-regulated in pancreatic tumors compared to the normal adjacent tissues. This may be because the FS isoform is the substrate of the NMD and is not stable after being spliced. Or there may be other alternative splicing that affects the measurements. Therefore, I could not measure the actual consumption rate of the pre-mRNA by the FS isoform in this assay.

4.2.3 Transient knock down of the expression of the WT SMC1A could not induce the tumorigenicity of the HPED6 cell.

Down-regulated expression of SMC1A was frequently observed in human pancreatic tumors. To test if the down regulation of the SMC1A could induce tumorigenicity, I first tried using siRNA to transiently "knock-down" SMC1A expression in HPDE6 cells. HPDE6 cell were established from primary normal pancreatic duct epithelial cells by transducing HPV16-E6E7 gene. The HPDE6 cell exhibits near normal genotype and phenotype of the pancreatic duct epithelial cells. The proliferation of the HPDE6 cells is anchorage dependent, and they

are non-tumorigenic in SCID mice. Two siRNA of human SMC1A were designed and I tested their efficiency at "knocking-down" the gene through RT-PCR techniques after 48 hour transduction. The GAPDH siRNA was included as the positive control for the procedure, and the non-target siRNA was included as the negative control for the tumorigenicity induction. The RT-PCR results demonstrated that all of the three siRNA could specifically knock down the targets in 48 hours, and the non-target siRNA had the same expression of the tested genes as the non-treated cells (Figure 4.4). All of these cells were treated with siRNA for 72 hours and then were expanded. The tumorigenicity of these cells was measured by the anchorage independent growth (AIG) assay. I just did one plate for each siRNA treatment and no-treatment cells. The AIG assay demonstrated that there was only a few colonies more in SMC1A siRNA treated cells compared to the negative control cells. Overall the number of anchorage independent growth colonies was low. This result and other reports suggested that the transient knock down of SMC1A could not efficiently induce the tumorigenicity.

4.2.4 Stably knock down the expression of the WT SMC1A could induce the tumorigenicity of BALB-3T3 cell.

The transient knock down of SMC1A for 72 hours may not be enough time for cells to take on characteristics of tumorigenicity. I then tried to stably "knock-down" SMC1A in BALB-3T3 mouse embryonic cells by shRNA through the lentivirus expression system. BALB-3T3 mouse embryonic cell have no

tumorigenicity in SCID mouse. Switching the target cell lines from the human to mouse system helped with the future in vivo analysis. Two efficient shRNA targets were selected and cloned into the lentivirus expression vector. These two shRNAs were located at exon21 and exon23 of the mouse SMC1A mRNA and named smc38 and smc75, respectively. Two BALB-3T3 cell clones with stable expression of smc38 and smc75 were isolated and established, as well as, the BALB-3T3 cell clone with stable expression of a non-target shRNA as the negative control. The efficiency of the SMC1A mRNA "knock-down" in these three cell lines was analyzed using RT-PCR with the primer set that could amplify a fragment in exon23 and exon24, as well as the non-treated BALB-3T3 cells (Figure 4.4). This demonstrated that about 50% of the WT SMC1A mRNA was "knocked-down" in both smc38 and smc75 clones. It was reported that 100% deficiency of SMC1A in yeast was lethal, so that the 50% knock down should be the most efficient knock-down achieved. Heterozygous deletion of SMC1A in yeast is sufficient to induce chromosome instability [211]. The tumorigenicity of these two cell lines was analyzed by the anchorage independent growth assay, as well as the negative control cell line. I also analyzed the tumorigenicity of the polyclonal smc38 BALB-3T3 cells (Figure 4.5). $5x10^3$ cells were plated in the agarose medium and cultures were kept for 3 weeks. The cell clones greater than 50um were counted. Both polyclonal and single clones of smc38 BALB-3T3 cells had more anchorage independent growth compared to the negative control cells. This clearly demonstrates that the knock-down expression of SMC1A could

induce tumorigenicity of normal cells. I also tried to characterize the in vivo tumorigenicity of the smc38 BALB-3T3 by subcutaneously inoculating the cells into normal BALB/c mice. The smc38 BALB 3T3 cell failed to develop into a tumor in normal BALB/c mice. This may be caused by the immune rejection induced by the expression of the lentivirus protein in the cells. Further investigation should use the immune deficient mice for the in vivo tumorigenicity evaluation.

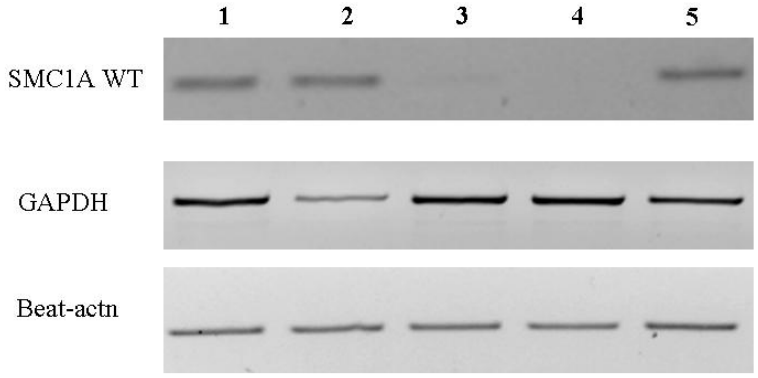


Figure 4. 4 RT-PCR analysis of the SMC1A knock down in HPDE6 with siRNA. The HPDE6 cells were treated with the different siRNA as indicated for 48 hours and followed by the RT-PCR analysis for the mRNA level of the different genes. 1. No-target siRNA treatment was the negative control for the tumorigenicity; 2. GAPDH siRNA treatment was the positive control for the experiment procedure; 3 and 4. smc05 and smc08 siRNA treatment respectively; 5. no-treated cells

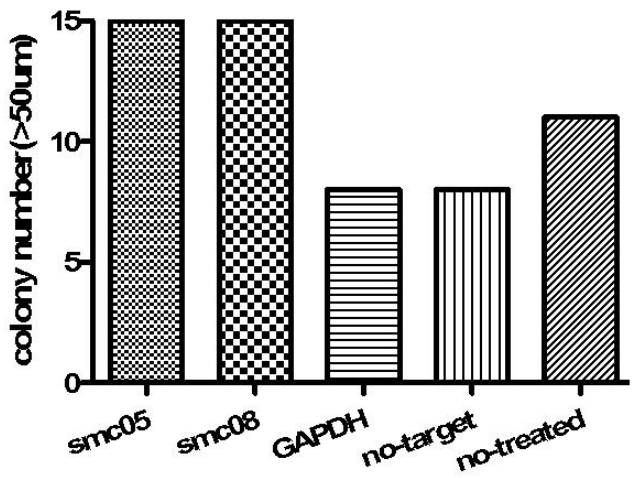


Figure 4. 5 Anchorage independent growth assay. 1x104 of each treated HPDE6 cells were plate in the argarose medium and incubated for 16 days. Colony size >50um were counted.

4.2.5 Detection of the SMC1A FS truncated protein

The SMC1A FS isoform was frequently detected in the different tumors. To test the hypothesis that FS isoforms of SMC1A have the ability to promote tumor development, I first designed the experiment to detect the expression of the truncated protein encoded by the FS isoform in tumors. We generated high titer anti-human SMC1A FS peptide rabbit serum by immunizing rabbits with the 17 amino acid human SMC1A FS peptide conjugated to KLH. Both the ELISA and western blot showed that the anti-serum has cross reactivity to the mouse SMC1A FS peptide (Figure 4.6). As mentioned above, the predicted molecular weight of the human truncated SMC1A FS protein is 6.08KD and 5.98KD in humans and mice, respectively. Both of them contain the same initial 37 amino acids on the N-terminus of the WT SMC1A protein, and there is a 2 amino acid difference in the C-terminus FS peptide tails. I performed a western blot (WB) of four human

breast tumor cell lysates and a mouse melanoma tumor cell lysate with the affinity purified rabbit anti human SMC1 FS antibody. All 5 tumor cell lysates showed a specific detected band around 16KD (Figure 4.7).

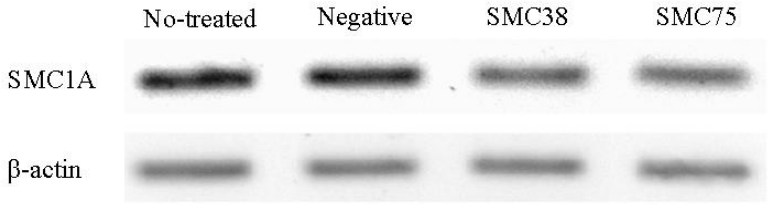


Figure 4. 6 RT-PCR analysis of the expression SMC1A stable knock down in BALB-3T3 cell. The fragment in the exon23 and exon24 were used to evaluate the efficiency of the SMC1A knock down by shRNA through the lentivirus expression system. The negative cell line expressed the no-target shRNA, both SMC38 and SMC75 cell lines expressed shRNA that target different sequence of the WT SMC1A.

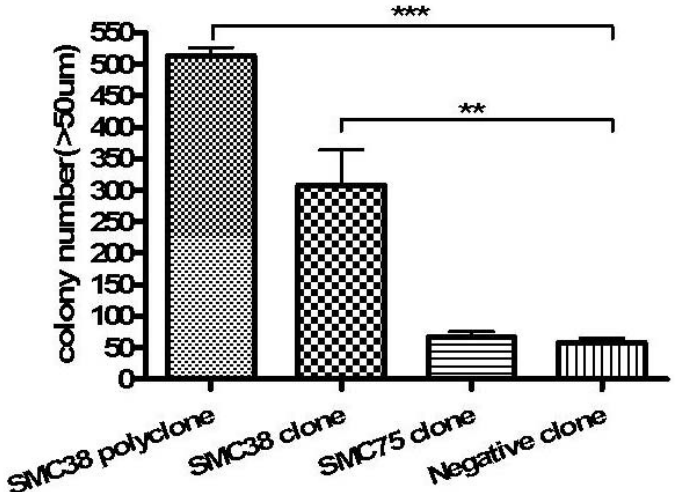


Figure 4. 7 Anchorage independent growth assay. 5k of each transfected BALB-3T3 cells was plated in the argarose medium and incubated for 3 weeks. Colony size >50um were counted. Data presented were from three repeated experiments with duplication of each cell line in each experiment. Each bar was the average from 6 plates and error bar is the standard error. ***: $p<0.0001$; **: $p<0.005$

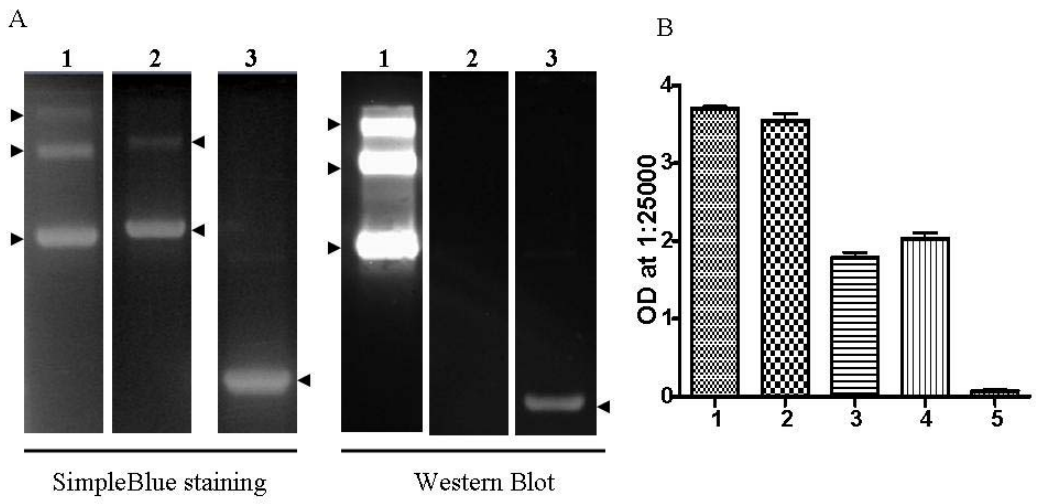


Figure 4. 8 Analysis of cross reaction of rabbit anti-human SMC1A FS peptide anti-serum to mouse SMC1A FS peptide. A. Western blot analysis of the cross reaction of the rabbit anti-human SMC1 FS peptide anti-serum to the GST fused mouse SMC1A FS peptide protein. Left: the SDS-page with simple staining of the protein fractions. Arrows indicate the detected fractions. Right: the WT analysis with the rabbit anti-human SMC1A FS anti-serum. Arrows indicate the detected fractions that specifically recognized by the anti-serum. 1. 1ug BSA coupled human SMC1A FS 17aa; 2. 1ug BSA; 3. 2ug GST-fused mouse SMC1A FS 27aa. B. ELISA analysis of rabbit anti-human SMC1A FS anti-serum, plate was coated with 50ul of each peptide at 10ug/ml. 1. human SMC1A FS 17aa; 2. human SMC1A FS 27aa; 3. mouse SMC1A FS 17aa; 4. mouse SMC1A FS 27aa; 5. human 1-78 FS peptide 21aa, as negative control.

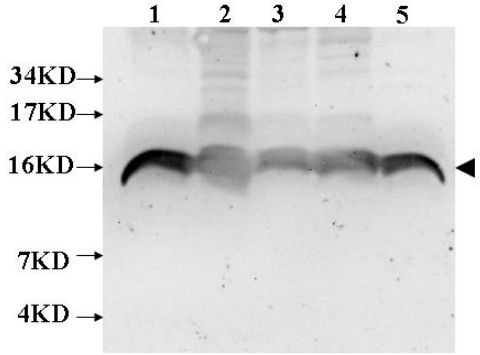


Figure 4. 9 WB of human and mouse tumor cell lysate with affinity purified rabbit anti-human SMC1A FS antibody. Load 100ug lysate of each tumor cell line. 1. CRL2351; 2. CRL2335; 3. CRL7253; 4. CRL2326; 5. B16F10. Arrow indicates the 16KD fraction that was specifically recognized by the rabbit anti-human SMC1A FS peptide anti-serum.

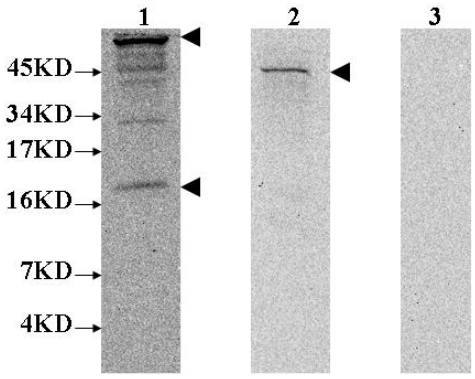


Figure 4. 10 WB of human breast tumor cell CRL2351 lysate with the commercial rabbit anti-human wild type SMC1A N-terminal antibody. 100ug lysate of each sample was loaded for WB. 1. the commercial anti-human SMC1A 1-288aa antibody, top arrow indicates the WT SMC1A; bottom arrow indicates the 16KD fraction; 2. rabbit anti-human beta-actin antibody, arrow indicates the beat-actin expression in the lysate for the positive control; 3. HRP labled goat anti-rabbit IgG 2nd antibody for the control of the secondary antibody background.

To confirm that the specifically detected 16KD fraction contained the truncated SMC1A FS protein, I also performed a WB of human breast tumor cell lysate with a commercial polyclonal antibody that can recognize WT human SMC1A N-terminal 1 to 288 amino acids. This commercial antibody should recognize both the whole WT SMC1A protein and the truncated FS protein, which contains the first 37 amino acids of the WT SMC1A protein. This antibody can also recognize the mouse wild type and truncated SMC1A proteins since both human and mouse share the same protein sequence that is encoded by exon1. The result showed that the commercial antibody also could specifically detect the 16KD band in the lysate of human breast tumor cell line CRL2351, as well as the full length of SMC1A protein (Figure 4.8). The specifically detected band by both antibodies was 10KD bigger than the predicted protein size. It was possible that the truncated protein is modified.

To further investigate this 16KD fraction, I used the B16F10 tumor cell lysate. Protein glycosylation is one of the most common post-translation modifications. To test if the 16KD fraction is glycosylized, I treated the total B16F10 cell lysate with enzymatic deglycosylation with the deglycosylation kit and chemical deglycosylation with trifluoromethanesulfonic acid (TFMS). Both treatments did not change the mobility of the 16KD fraction in WB analysis (Figure4.9). This indicated that glycosylation did not affect the mobility of the detected band.

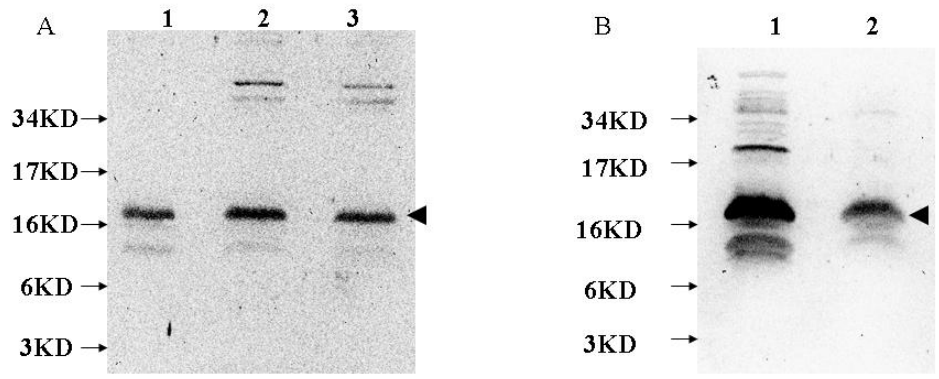


Figure 4. 11 WB of deglycosylation of B16F10 tumor cell lysate with the rabbit anti-human SMC1A FS peptide antiserum. A. WB of enzymatic deglycosylation cell lysate. 1. 10ul untreated lysate; 2. 10ul of 4 hours enzymatic treated lysate; 3. 10ul of 8 hours enzymatic treated lystae. B. WB of trifluoromethanesulfonic acid (TFMS) chemical deglycosylation cell lysate. 1. 20ul untreated cell lysate; 2. 20ul TFMS treated cell lystae. Arrows indicate the 16KD fractions.

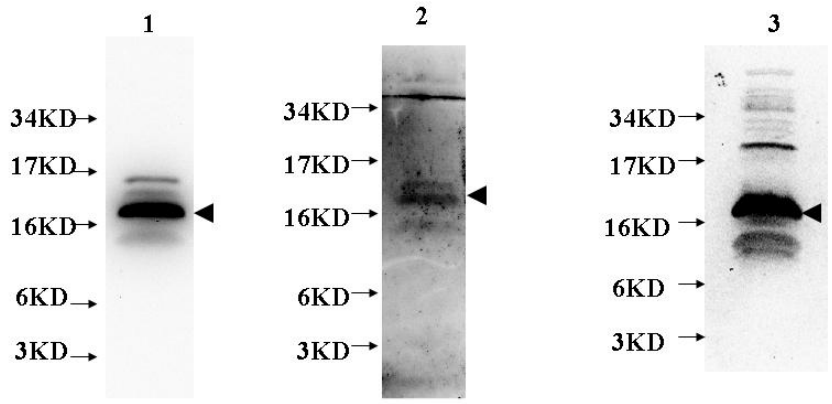


Figure 4. 12 WB of total B16F10 denatured lysate (20ul). Rabbit anti-SUMO2/3 antibody (lane 1); rabbit anti-human wild type SMC1A N-terminal (1-288aa) antibody (lane 2); rabbit anti-human SMC1A FS antibody (lane 3). Arrows indicate the 16KD fractions.

Before further analysis of the 16KD fraction, I searched the other possible post modifications that could add about 10 KD mass to the target proteins. It was turned out that the SUMOylation is the most likely post-translational modification which can add about a 12KD mass to the target proteins. The WB analysis demonstrated that anti-sumo2/3 antibody, anti-human SMC1A N-terminal antibody and anti-human SMC1A FS antibody can recognize the same 16KD fraction of the B16F10 lysate (Figure 4.12). This indicated the 16 KD fraction of from the tumor lysates contain the truncated SMC1A FS protein which could be SUMOylated by sumo2 or sumo3.

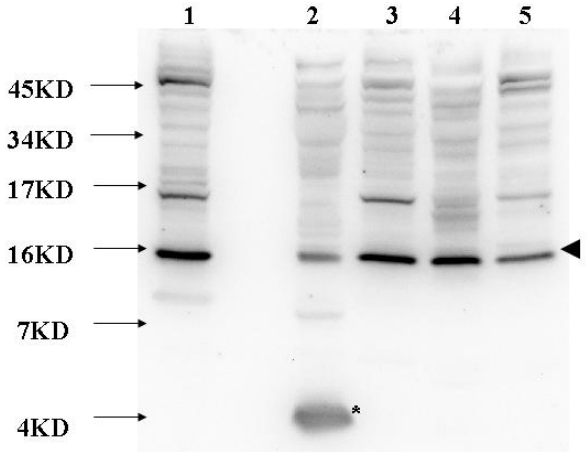


Figure 4. 13 WB of B16F10 denatured lysate and normal C57BL6 tissues lysates (200ug each) with rabbit anti-human SMC1A FS antibody. 1. B16F10 lysate; 2. heart lysate; 3. intestine lysate; 4. lung layeste; 5. spleen lysate. Arrow indicates the 16KD fractions. Star indicate the possible un-modified SMC1A FS truncated protein in heart lysate

It was demonstrated that the SMC1A FS transcript was low in the normal tissues, and was barely detected by RT-PCR in some normal tissues from mouse, such as lung, intestine and heart. To investigate the different expression of the truncated protein between tumor and normal tissues, I performed the WB analysis of some normal mouse tissue lysates with anti-human SMC1A FS antibody (Figure 4.13). Surprisingly, the WB result exhibited that all of the normal tissues contain the same 16KD fraction as the B16F10 tumor cells. The intensities of the detected 16KD fractions were similar among B16F10 lysate, intestine lysate and lung lysate. Both the intenstine lysate and spleen lysate exhibited the same WB pattern as the B16F10 lysate. The normal heart lysate also exhibited the specific detection of the 4KD band, which is approximately the size of the SMC1A FS isoform.

To directly confirm if the 16KD fraction contain the truncated SMC1A FS protein, I tried different methods. The immunoprecipitation of tumor cell lysate with the FS antibody failed to pull down the 16KD fraction. Additionally, in-gel trypsin digestion of the 16KD fraction followed by mass spectrum analysis failed to detect the truncated protein as well.

4.2.6 The truncated protein encoded by the SMC1A FS transcript is located in the nucleolus

To further investigate the expression of the truncated protein in both tumor and normal tissues, we collaborated with Dr. Atul K. Tandon at NeoBiomarkers Inc. and performed the immunohistochemistry (IHC) analysis of primary tumor and normal tissue sections with the rabbit anti-SMC1A FS serum (Figure 4.14). The nucleolus of both tumor and normal tissues were stained by the anti-serum, as well as the cytoplasm of some tissue structures. Both of the squamous cell carcinoma of the skin and the esophagus exhibited the clear membrane staining by the specific rabbit anti-serum.

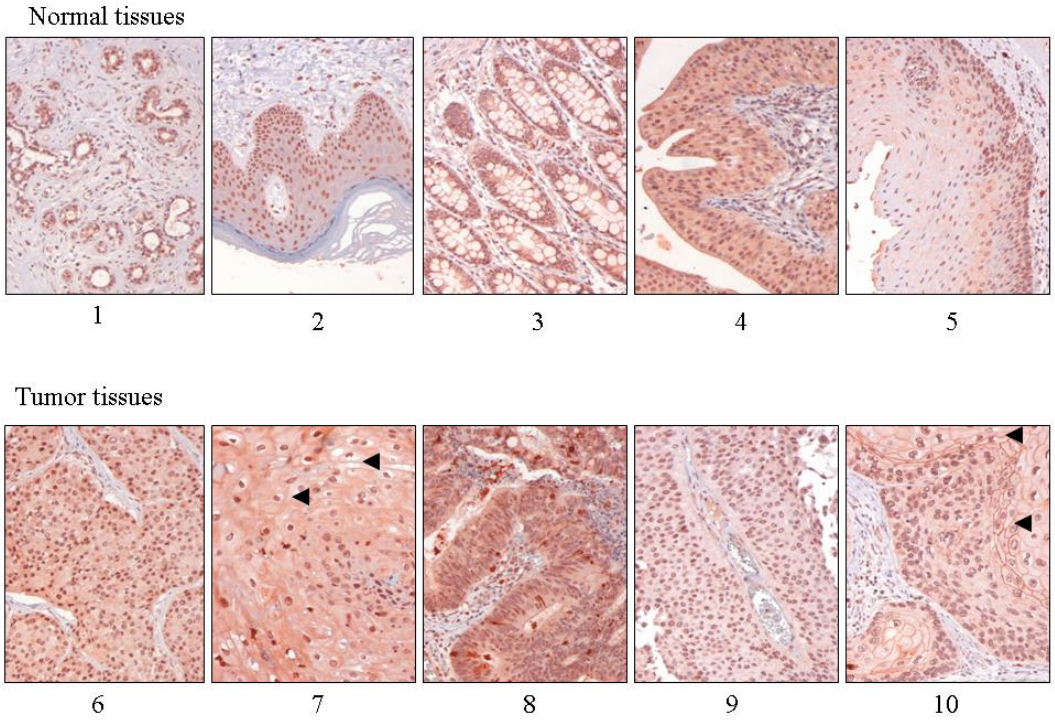


Figure 4. 14 Immunoehistiochemistry analysis of formalin-fixed human normal tissues and primary tissues with rabbit anti-human MSC1A FS anti-serum. 1. normal breast; 2. normal skin; 3. normal rectum; 4. normal bladder; 5. normal esophagus; 6. breast carcinoma; 7. squamouse cell carcinoma of skin; 8. rectum adenocarcinoma; 9. transitional cell carcinoma; 10. squamouse cell carcinoma of esophagus. blue: hemotoxylin staining nuclear; red/brown: antibody staining. The experiments were performed by Dr. Atul K. Tandon at NeoBiomarkers Inc

The WT SMC1A usually forms cohesin and localizes in the cell nucleolus. Although the truncated protein was detected in both tumor and normal cells, it is possible that the expression of the truncated protein increases in tumor cells and promotes the tumor development by affecting the function of the WT protein in the cell nucleolus. It has been reported that the WT SMC1A protein will accumulate at the sites of the double stranded DNA break (DSB) of the DNA. To test if the truncated protein accumulated at DSB sites, we

collaborated with Dr. Yaron Galanty (The Wellcome Trust/Cancer Research UK Gurdon Institute) and performed the laser micro-irradiation of the U2OS cells (human osteosarcoma cell) and followed the immunofluorescence (IF) assay (Figure 4.15 A). IF results of the non-treated U2OS cells demonstrated that the truncated protein was located in the nucleolus of the cells. This was consistent with the IHC results. IF assay of the mitotic cell exhibited that the truncated protein was excluded from the chromosomes at anaphase (Figure 4.15 B). However, the truncated proteins were not accumulated at the sites of the DSBs (Figure 4.15A).

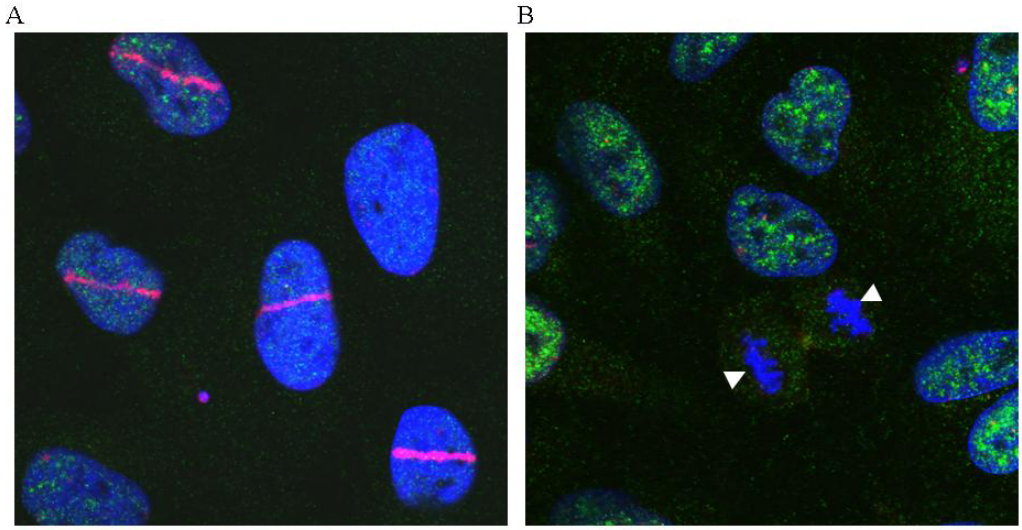


Figure 4. 15 IF assay of the cellular location of the truncated protein of the SMC1A FS isoform in U2OS cell, human osteosarcoma cell line. A. laser micro-irradiation of the cells before the IF staining. B. IF staining of the mitotic cells (indicated by the arrows). Blue: indicates nucleolus with DAPI staining; Red: indicates DSB sites by laser irradiation with anti-γ-H2AX antibody; Green: indicates the SMC1A FS truncated protein with the affinity purified rabbit anti-serum against human SMC1A FS peptide (17aa). (Assay were performed by Dr. Yaron Galanty, The Wellcome Trust/Cancer Research UK Gurdon Institute)

4.2.7 Evolutionary evidence of the truncated SMC1A FS protein may have function in normal cells

The detection of Sumolyation of the SMC1A FS truncated protein and the nucleolus localization of this truncated protein in normal cells indicated that the SMC1A FS protein may have nucleolus function in normal cells. SMC1A is a highly conserved gene in eukaryotes. It is interesting that the SMC1A FS peptides are also highly conserved between human and mouse (Table 1.1). I explored an evolutionary analysis of the SMC1A FS protein to see if there was any evidence to support the suggestion.

Table 4. 1 Conservation analysis of the SMC1A DNA sequence. The identities of different DNA fragments of 5 eukaryote species to Homo sapiens were analyzed with NCBI Blast. Higher identity presents higher conservation. P values of the paired t test of identities of DNA fragment encoding the SMC1A FS peptide (exon4-fs) verse entire CDS and the downstream of the exon4-fs of the exon (exon4-rest) were less than 0.05.

		Identity to Homo sapiens NM_006306.2 (perscent)			
Species	Accession No.	Entire CDS	Exon3	Exon4-fs	Exon4-rest
Xenopus laevis	BC046691	81	78	88	84
Mus musculus	NM_019710.2	92	93	93	89
Rattus norvegicus	NM_031683.1	92	92	95	89
Bos taurus	NM_174614.1	94	95	98	90
Canis lupus	XM_538049.3	94	96	98	93

The conservation of a gene is derived from the evolutionary selection. A gene with a critical cellular function is usually highly conserved across different species [228]. It is also true for an important domain within a gene. For example, the N-terminal Walker A domain is highly conserved in the SMC super family genes [229]. Therefore, the highly conserved DNA fragment of a gene may encode an important domain. I analyzed the conservation of the DNA fragments

encoding the SMC1A FS peptide (exon4-fs) and the two connected fragments: the exon 3 which is upstream of the exon-fs and the downstream of the exon4-fs of the exon 4 (exon4-rest), as well as the conservation of the entire coding DNA sequence (CDS) of the SMC1A (Table 4.1 A). There is no significant difference comparing the DNA identities among the entire CDS, exon3 and exon4-rest across the 5 eukaryote species. This indicates that both upstream and downstream DNA fragments of the exon4-fs are under similar evolutionary selection as the entire CDS. It is interesting that the exon4-fs has significantly higher identities than the entire CDS and the exon4-rest. Although the average of identities of the exon4-fs across the 5 species is higher than the exon3, there is no significant difference of the conservation between the exon3 and exon4-fs. The exon3 encodes the first coiled-coil region after the highly conserved N-terminal Walker A domain. This may increase the conservation of the exon3 and select for less difference of the conservation between exon3 and exon4-fs. The DNA conservation analysis compared to the other sequences, however, indicates that the exon4-fs may encode a functional domain. I further analyzed the conservation of the peptides encoded by these three DNA fragments (Table 4.2). The peptides encoded by the primary reading frames of the exon3, exon4-fs and exon-rest are 100% identical in 4 of the 5 selected eukaryote species. The entire exon4 encodes part of the coiled-coil region and there is no special annotated domain in this region of the WT protein. This indicates that the higher identity of the exon4-fs may not be evolutionally derived by the primary reading frame. However, the 3rd

reading frame of the exon4-fs has significantly higher identity than the other two fragments (Table 4.2). This is the reading frame that encodes the SMC1A FS peptide. It is interesting that there is no significant difference in the conservation of the primary and 2nd reading frames between the exon3 and exon4-fs. This suggests that the higher conservation of the exon4-fs may be derived by the evolutionary selection of the peptide encoded by the 3rd reading frame. The peptide alignment of the FS peptides from 5 eukaryote species shows that there is a highly conserved peptide fragment: CHEEPQ. In summary, these evolutionary conservation analyses indicate that the SMC1A FS truncated protein may have the cellular function in normal cells. It is necessary to bioinfomatically analyze the conservation of the FS fragment in more species and possible functional motifs of the FS peptide for the further investigation of the biological function of the SMC1A FS truncated protein.

Table 4. 2 Conservation analysis of peptides encoded by different reading frames the SMC1A DNA fragments. A. The identities of different peptides of 5 eukaryote species to the correspond peptide of Homo sapiens were analyzed with NCBI Blast. Higher identity presents higher conservation. P values of the paired t test of identities of the third reading frame of the exon4-fs verse the exon3 and exon4-rest were less than 0.05. B. The comparision of the SMC1A FS peptide sequence of different species.

A

	Identity of different reading frame of DNA fragments of the SMC1A (%) to Homo sapiens								
	Primary reading frame			Second reading frame			Third reading frame		
Species	Exon3	Exon4-fs	Exon4-rest	Exon3	Exon4-fs	Exon4-rest	Exon3	Exon4-fs	Exon4-rest
Xenopus laevis	92	100	100	42	65	53	43	59	50
Mus musculus	100	100	100	78	76	67	76	76	64
Rattus norvegicus	100	100	100	75	88	69	76	82	66
Bos taurus	100	100	100	83	94	71	85	94	68
Canis lupus	100	100	10	89	94	82	88	94	77

B

Homo sapiens	CCGIYCHEEPQREDSSI
Xenopus laevis	GGGVHCHEEPQRAYSSV
Mus musculus	CSGVYCHEEPQGEDSSV
Rattus norvegicus	CSGIYCHEEPQGEDSFI
Bos taurus	CCGIYCHEEPQREDSSF
Canis lupus	CCRIYCHEEPQREDSSI

4.3 Materials and Methods

4.3.1 Quantitative PCR

Real-time PCR were performed with REALMASTERMIX kit (Eppendorf, Germany), follow the manufacturer's protocols. PCR condition: 95^{o}C 2 min; (95^{o}C 15 sec; 60 ^{o}C 15 sec, 68 ^{o}C 20sec)x40 cycles; 95 ^{o}C 15sec.

4.3.2 siRNA assay

Pre-designed human SMC1A and GAPDH siRNA were order from Dharmacon (Thermo Fisher Scientific, Lafayette, CO). The transient SMC1A

knock down assay was performed in 6 wells plate with the manufacturer's protocol. Each siRNA treatment was duplicated. After 48 hours treatment, one of the duplicated siRNA samples was use for RT-PCR analysis to measure the knock down efficiency, another one was expended and use for the AIG assay to measure the tumorigenicity.

Smc05: 5'-GGACAGCUCUAUUUGAAGAUU

Smc08: 5'-GAACUGGCCUCAAAGAACAUU

4.3.3 shRNA assay

The shRNA assay was performed with BLOC-iT^{TM} Lentiviral Pol II miR RNAi Exression System (Life Technologies, Carlsbad, CA). Two targets of the mouse SMC1A gene were designed and synthesized for cloning, as well as a no-target sequence:

SMC38:

TGCTGAGGAGCTACACAGTTGTAGTTGTTTTGGCCACTGACTGACAAC

TACAAGTGTAGCTCCT;

SMC75:

TGCTGAGCCTTGGTGTAGAATTCCTCGTTTTGGCCACTGACTGACGAG

GAATTACACCAAGGCT;

Neg:

TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTC

TCCACGCAGTACATTT

Briefly, the synthesized oligos were cloned into Gateway vector system and finally constructed into pLenti6/V5 Expression vector and sequence confirmed. Co-transfect the pLenti6/V5 Expression vector with ViraPower Pawer Packaging Mix into 293FT cells and produces the lentivirus. Transduce the lentivirus into BALB-3T3 and select transfected cells with blasticidin. After the selection, expand the cells and then use part for sorting single clones and keep some as a population. The SMC1A knock down was measured in the population cells with RT-PCR analysis.

4.3.4 Anchorage independent growth assay

Prepare the plate

Prepare 2x DMEM with 5% FBS and pen/strep and keep in a 37°C water bath. Make a 2X 0.8% agarose stock in sterile water and autoclave. When the agarose cools to 50°C, mix with equal volume of the 2x medium. Quickly add 2.5ml mixed medium to 60mm plate. Keep plates at 4°C.

Prepare the assay

Make 0.75% agarose stock in sterile water and autoclave. Then put in a 45^{o}C water bath. Pre-heat the 2x medium and plate at 37°C. Prepare cells and adjust to 2x10^{4}/ml in 1xDMEM medium and keep at 37°C. Mix 5ml 2X DMEM+4ml 0.75% agrose+1ml cells and quickly pour 3ml of the mixed over the bottom layer of the plate. Duplicate the plate. It will be 0.3% aragose with 5,000 cells in each plate. After the top layer cools, add 1ml complete 1x DMEM on the top. Culture for 3-4 weeks and count the colonies.

4.3.5 Enzymatic deglycolyzation assay

Follow the manufacturer's protocol with the Protein Deglycosylation Mix (NEB, Ipswich, MA). Dissolve in 100ug desalted B16F10 lysate into 18ul water and add 2ul denaturing buffer and heat to 100°C for 10 minutes for the denaturing reaction. Then add 5ul G7 reaction buffer, 5ul 10% NP40, 15ul water and 5ul deglycosylation enzyme cocktail and mix gently. Incubate the reaction at 37 °C for 4 hours and 8 hours. Store the treated samples at -80°C.

4.3.4 Chemical Deglycosylation with Trifluoromethanesulfonic Acid (TFMS)

The salt free and lyophilized B16F10 cell lysate was prepared. Add 150ul pre-cooled TFMS into the 1mg lyophilized cell lysate. Gently shake the sample to dissolve the lysate. Incubate the sample on ice for 25 minutes. Then add pre-cooled 60% Pyridine Solution drop by drop up to the 300ul. Check the pH with pH paper until it reachs around 6. Use the desalt column to remove the TFMS salt. Store the samples at -80°C.

4.4 Discussion

Genome instability is a fundamental hallmark of the molecular alternations of the tumor. It could drive the tumor initiation and progression [106]. Mutations of the genes involved in genome integrity increase the risk of the cancer. For example, mutation of the mismatch repair gene hMSH2 is related with colon cancer. People with the inherited mutation of hMSH2 gene have a high risk of the colorectal cancer because of the microsatellite instability [230, 231].

SMC1A gene has multiple important functions to maintain genome integrity. The deficiency of SMC1A in tumors was observed in pancreatic cancer by genetic mutation and in AML patients by decreasing protein expression. Both the mutations and knock-down of the WT SMC1A could induce spontaneous genetic instability [210, 227]. These previous studies indicate that SMC1A could be involved in tumor initiation and progression. However, the genetic mutations of SMC1A are rarely identified in different cancers [232]. Most identified SMC1A mutations in cancer were mis-sense mutations, which still could keep some the primary function of SMC1A [225]. Missense mutations and small in-frame deletions were also identified in CdLS, a developmental disorder. A severe mutation of SMC1A could reduce the fitness of the cell and even cause cell death [210, 211]. This may be the major cause of low frequency mutations of SMC1A identified in tumors. There was also no published evidence about the low SMC1A expression in tumors based on mRNA quantification. This indicated that the deficiency of SMC1A in tumor cells, such as lower SMC1A protein detection in AML patients, may be caused by some other pathway than genetic mutations.

Our findings in this study suggested that the deficiency of the SMC1A RNA in tumor cells is mainly caused by the mis-regulated splicing and this is common in different type of tumors. The mis-regulated splicing in cancers has been well documented. Splicing errors promote tumorigenicity either by splicing an isoform with oncogenetic activity or by simply decreasing the tumor suppressor isoform through consuming the pre-mRNA with nonfunctional

isoforms, or both [200, 208]. We found that there was mis-regulated splicing of the SMC1A in different tumor cells. Different splicing isoforms of the entire SMC1A were detected in tumor cells. Most of them were in frame deletions, which could encode proteins with residual functions and affect the WT protein function or even have negative functions.

We also found a unique FS isoform in different tumors. This was caused by exon2 and exon3 skipping. The SMC1A FS isoform and its relative WT isoform were deeply studied. I found that the FS isoform was frequently increased in pancreatic tumors, while the relative WT isoform was frequently decreased. The increasing of the FS isoform was also detected in multiple tumor types. This was the first evidence of decreasing SMC1A WT mRNA isoform in tumor cells and this may be due to splice switching to the SMC1A FS isoform. There have been no other studies that have shown the decrease of SMC1A mRNA in tumors [226]. And there was no correlation found between SMC1A mRNA levels, and the lower SMC1A protein level. This may be caused by using different target fragments of the SMC1A mRNA for quantification.

It has been published that the deficiency of the SMC1A, either caused by mutation or siRNA treatment, could spontaneously induce the genetic instability and aneuploidy of normal cells [227]. There was no direct evidence of the correlation between the deficiency of the SMC1A and tumor development. The transient SMC1A knock down by siRNA did not efficiently induce tumorigenicity in HPED6 cells, which was consistent with the previously published study.

However, the stable SMC1A knock down in BALB-3T3 cells successfully induced significant tumorigenicity. This was the first evidence that directly showed tumor initiation by deficiency of SMC1A. Our previous study looking at the different stages of tumor development in FVB/N-NeuT, demonstrated that the mis-regulated splicing of SMC1A happened at early stages of tumor development along with the expression of the tumor driven gene, Rat Her2. All of our findings indicate that the constant deficiency of SMC1A by mis-regulated splicing promotes the initiation and progression of different type of tumors.

The increase of SMC1A FS isoform was detected in multiple tumors. This FS transcript contains a PTC, and is a classical target of the NMD. The selective protection of this FS isoform from the NMD indicates this FS isoform may have unique functions to promote tumor development, aside from the side product of the mis-regulated splicing. It was interesting that the truncated protein encoded by the SMC1A FS isoform was detected in both normal tissues and tumors and was sumoylated with SUMO2/3 by WB analysis. The IHC assay and IF assay with the anti SMC1A FS peptide antiserum also showed the expression of the truncated protein in tumor and normal cells. These two assays also revealed that most of the truncated protein was located at the nucleolus of both tumor and normal cells. The SUMOylation has a variety functions for protein stability maintenance, protein cellular localization and transcription [233, 234]. The SUMOylation of the target could stabilize the protein by preventing the ubiquitination [235]. That may cause the accumulation of the SMC1A truncated protein in normal cells, although the

transcript level of the FS isoform are low in normal cells. The SUMOylation also could regulate protein cellular localization [236, 237]. Both mammalian SUMO2 and SUMO3 were detected at the cell nucleolus, where the SMC1A truncated protein localized. This indicates that the SMC1A truncated proteins are stabilized and localized at the nucleolus by SUMOylation. The evolutionary analysis of the exon4-fs showed that the FS peptide encoded by this fragment is highly conserved across five eukaryote species. This indicates that the SMC1A FS truncated protein may have cellular function in normal cells.

In summary, this study supports the concept that the WT SMC1A acts as a tumor suppressor gene, and also suggests that SMC1A FS isoforms are expressed and produce their effects within the cell nucleolus. The mis-regulated SMC1A FS isoform is common in a variety of cancer types and could have oncogenetic function to promote tumor development. However, further characterization of both WT and FS isoforms is still necessary to provide solid evidence of the isoforms' function in tumor development.

CHAPTER 5

SUMMARY

Cancer is one of the most serious global diseases that affects human society in both health and economy. Although we have achieved dramatic developments against cancer over the last hundreds years, we still can not efficiently control this disease. The development in cancer prevention, especially in the cancer immunoprevention, has shown this is the most cost-effective long term aspect for global cancer control. However, research on cancer immunotherapy needs much more efforts to further develop.

Our center has focused on cancer immunoprevention for years. In this thesis I described our systematic strategy for general cancer prevention through variety of immunological methods.

Prophylactic primary and metastatic cancer vaccines based on the tumor specific and related FS antigens is one of the most important concepts we developed for the cancer immuneprevention. The goal of this concept is to develop a general cancer vaccine that could prevent healthy people from developing different types of cancer. Our center has identified a list of tumor specific and related FS antigens that are frequently presented in different human cancers and mouse tumor models. *In silica* analysis demonstrated that the list of the FS antigens is sufficient to cover major cancer types and most HLA types in the population (Lee and Johnston, in preparation). I tested the concept with FS antigens in a variety of mouse tumor models, including transplant mouse tumor

models: 4T1/BALC/c and B16F10/C57BL6; and transgenic mouse tumor models: BALB-NeuT and FVB/N-NeuT. Although there is no qualified epitope base on the *in silica* prediction of these FS antigens, the prophylactic cancer vaccines with different FS antigens, especially the SMC1A FS antigen, have shown significant inhibition of the development primary tumors in all of four mouse models, and it also has shown the potential to inhibit the tumor metastasis incidence in the 4T1/BALB/c model.

The concept of innate stimulation to prevent tumor onset adds another layer of the cancer immunoprevention. The clinical applications of this concept have been tried for years; however it has been limited by use of poorly defined components or the toxicity. I tested this concept with a viral immune modulator B2L, which was identified in our Center recently [162]. Compared with the control group and the group vaccinated with a known immune adjuvant CpG and GMCSF, the vaccination of B2L significantly inhibited tumor development in the BALB-NeuT model. All of the B2L treated mice behaved the same as other two groups and did not showed severe side affects. The significant efficacy of the B2L vaccination in tumor inhibition suggests the promise of the innate stimulation for the cancer prevention by identifying more components in future investigations.

Besides the immunoprevention, early cancer detection is also an important component in our systematic strategy of cancer prevention. Based on the studies of the tumor related FS antigen, SMC1A FS peptide, I discovered two types of cancer biomarkers: circulating FS transcripts and FS specific antibody. I also

developed two unique methods to improve the sensitivity and specificity of these two biomarkers.

The SMC1A FS transcript was frequently increased in the tumor cells by abnormal splicing. Using regular RT-PCR with the flanking primers, both WT and FS transcripts of SMC1A could be amplified and detected from the plasma of tumor bearing mice, while only the WT transcript was detected from plasma of the non-tumor bearing mice. Based on the electrophoresis analysis of the PCR products, the intensity ratio of the FS verses the WT was significantly correlated with the tumor size in the 4T1/BALB/c mouse tumor models. This indicated that the ratio of the two splicing isoforms not only could be used for early cancer detection, but also could be a unique biomarker for tumor progression. All kinds of tumor related alternative splicings with similar patterns are suitable for this unique detection method, including those tumor related FS splicings and in frame splicings. The ratio of the two splicing isoforms was amplified both in vivo and in vitro because of the size difference; therefore this method is more sensitive than other detection methods for cNA biomarkers. This detection method does not need an extra reference for quantification; therefore it is more robust and simple for large cancer screen.

The SMC1A FS antibody was detected by a set of mimotopes of SMC1A FS peptide. The epitope of the original SMC1A FS peptide was represented by a set of mimotopes on the peptide array. The sensitivity of the FS antibody detection was increased about 50 fold by using the set of the mimotopes. The

positive rate of the SMC1A FS antibody was 53.8% and 56.5% in breast cancer patients and pancreas cancer patients respectively. This was about 2 fold higher than currently reported positive rates of a single cancer related auto-antibody, such as anti-P53 auto-antibody, in cancer patients. The high sensitivity of SMC1A FS antibody detection is mainly contributed by efficient epitopes representation with the mimotopes. This method of using mimotopes could increase the detection sensitivity of all current antibody biomarkers of different diseases.

Based on the antibody biomarker, I also developed a concept of novel diagnosis for immune response based biomarkers; we name the "amplified diagnosis". The concept is straightforward: set up efficient memory immune response to disease specific antigens in disease free people through immunization. The adaptive memory immune response is more sensitive and reactive than the endogenous immune response to the exposure of the disease specific antigens. Therefore the immune response based biomarkers can be efficiently detected even by current detection methods.

Cancer is a complex and systematic disease. Efficient cancer control can not be accomplished by a simple method. The different concepts we developed are focused on tumor specific and related FS antigens. However, these strategies are also suitable for some of current tumor antigens and other related diseases. Based on different concepts I described in this thesis, we developed a systematic strategy of cancer immunoprevention: apply the innate stimulation and prophylactic cancer vaccine to generally prevent the tumor onset; apply the

amplified immune diagnosis and circulating RNA detection to early detect specific cancers for different early cancer treatment. Further development of this systematic strategy should make an important contribution to global cancer control.

Besides the development of applications for cancer control, my investigation of SMC1A also indicated that the wild type SMC1A may be down regulated in tumor cells by mis-regulated splicing which could induce the tumorigenicity of normal cells. On the other hand, the SMC1A FS truncated protein may be SUMOylated and accumulated in nucleolus of normal cells. Mis-regulated SUMOylated SMC1AFS protein may cause tumorigenicity in normal cells as well. Therefore, mis-regulation of both WT SMC1A and FS SMC1A may be the one of the early events of the process of normal cell transformation. Further investigation is necessary to confirm the hypothesis.

In summary, this thesis discussed the developments and tests of different concepts in cancer immunoprevention. And this thesis also suggested the tumor related FS antigens are a non-fully explored field which has important value for both translational and basic research of cancer control.

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APPENDIX 1

FS ANTIGEN DISCOVERY BY PROTEOMICS ANALYSIS

We are focusing on FS antigens for a prophylactic cancer vaccine. To discover tumor specific and related FS antigens, we systematically used bioinformatics, genetics, proteomics approaches to screen different tumor cells. Lee et.al. has described the strategy of using bioinformatics analysis to discover FS antigens (Lee and Johnston, in preparation). We also tried to use proteomics analysis to directly identify tumor related FS antigens from tumor cell surface and chaperone-rich cell lysate (CRCL).

The major function of molecular chaperones is in preventing protein aggregation. People also found that, in the case of cellular stress, chaperone proteins can recognize and bind to non-native structured proteins [239]. It has been shown that the CRCL purified from the 12BL tumor cell, a BCR-ABL$^+$ mouse leukemia cell line, could induce anti-tumor immune response in mouse and inhibit tumor growth [240]. Since the FS antigens we focused on are not correctly folded, truncated proteins and could accumulate in tumor cells, we hypothesized that CRCL could enrich the tumor related FS antigens and those antigens are potential candidates for a prophylactic cancer vaccine.

To identify the FS peptide in CRCL from 12B1 cell (CRCL and 12B1 cells were offered by Dr. Emmanuel Katsanis, University of Arizona), we used acid to dissociate the protein interactions and enriched the small peptides through 10KD or 3KD filters. The peptides fractions were analyzed by liquid chromatography-mass spectrometry. The potential FS peptides were identified by analyzing the MS data with our FS peptides database. A total 11 FS peptides were identified from CRCL of 12B1 (Johnston, Lake and Antwi, unpublished data).

I analyzed the potential T cell epitopes of list of FS peptides. Three of them have predicted T cell epitopes (Table AP1-1). These three FS peptides encoding sequences were constructed into pCMIVi-US and pCMVi-LS to test if they could inhibit the 12B1 tumor growth. Immunization of the pooled FS antigens showed the potential tumor inhibition in 12B1/BALB/c mouse tumor model (Figure AP1.1). However, both antibody and T cell response were undetectable in these mice. The genetic immunization methods and adjuvant were not optimized at the time I tested these three FS antigens. Further experiments with optimized immunization are necessary to verify the anti-tumor efficacy of these three FS antigens.

I did not detect the transcripts corresponding to these three FS peptide in 12B1 cells. As I discussed in chapter 2, these FS transcripts may be degraded by NMD. Inhibition of NMD in 12B1 cell can increase the possibility to detect these FS transcripts. Analysis the specific immune response to these FS peptides in mice immunized with CRCL of 12B1 cell also can confirm the exhibition of these FS peptides in 12B1 cells.

This experiment demonstrated the potential value of using proteomics analysis to screen the tumor FS antigens.

Table AP1. 1 FS peptides identify from LC/MS analysis of CRLC of 12B1 cell. (By Johnston, Lake and Antwi)

FS Peptides	Correspond Genes
HLDVPTFTICDQTHL	NM_145628.2_20(Usp11)
RIFNNGADLSGITEENAPLK	XM_978230.1_0(Serpina1d)
GIKAANTMFPMKCTSGQP	NM_054052.2_15(B3gnt5)
RNISTCPKLAGSTSPASM	NM_029674.1_18(Got1l1)
RPPSCDQNWCSVYSP	NM_207217.1_14(Itfg3)
WEHSCRRAGQLF	XM_622915.3_36(LOC547385)
HDCWKCIGRIY	NM_022317.3_19(Slc28a3)
VVAVKAPGFGDN	XM_982339.1_13(Hspd1)
VRGASGGVSDGSVQTPAEGRGAGQGQRAVTTDICPLLGL	NM_021327.1_1(Tnip1)
LDPSHPAAQQQGQQCSLREKRKLLLR	NM_027494.1_24(Zcchc8)
AWTGWAVAARPATAATCSRTLYWPARR	NM_010464.2_1(Hoxc13)

Table AP1. 2 T cell epitope prediction of three FS peptides

Peptides	Length (amino acid)	MHC I binding prediction (allel/binding shreshold/epitop score)
HLDV…	15	Kd 10mer/65/62
RNIS…	18	Kd 8mer/49/52 Dd 9mer/102/99
AWTG…	27	Dd 9mer/102/101

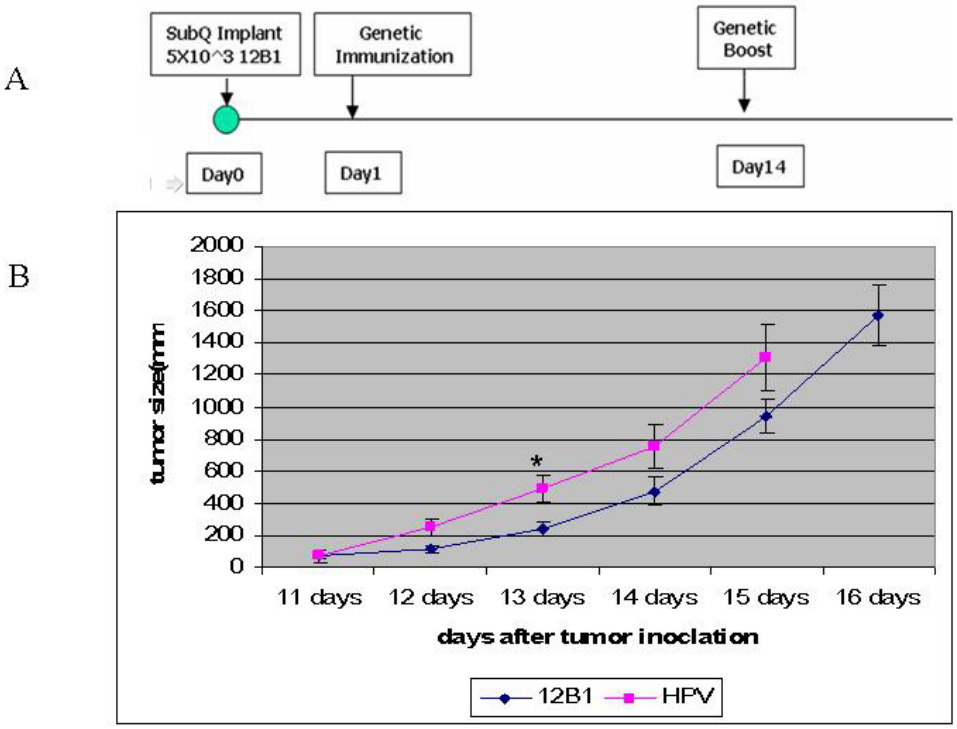
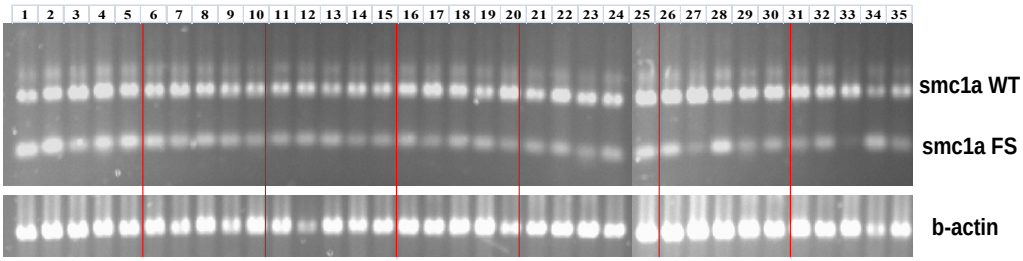


Figure AP1. 1 Protection analysis in 12B1/BALB/c mouse model. A. Experimental plan. 5x103 12B1 were inoculated into four to five weeks old BALB/C mice. The 12B1 group (n=15) was immunized with three pooled FS antigens; the HPV group (n=12) was immunized with HPV225a generated in our Center as the negative control). B. Tumor growth curve. Tumor sizes were measured daily after detecting palpable tumors. The data represents three individal experiments. *: t-test $P<0.01$

APPENDIX 2

RT-PCR ANALYSIS OF FS TRANSCRIPTS



1	MDA-MB-468	6	HCC1395	11	HCC70	16	HCC1937	21	MB157	26	ZR-75-1	31	Hs 605.T
2	SW 527	7	MDA-MB-453	12	UACC-893	17	HCC1428	22	Hs739.T	27	HS-362.T	32	BT474
3	HS 606T	8	HCC 1143	13	AU565	18	MCF-7	23	DU4475	28	BT-20	33	Hs 343.T
4	HCC1187	9	M4A4	14	HCC 1954	19	Hs-578.T	24	MDA-MB-231	29	HCC 2218	34	MCF-10-A
5	HCC1806	10	HCC1419	15	ZR-75-30	20	HCC1500	25	Cama-1	30	HCC 1599	35	HPDE6

Figure AP2. 1 RT-PCR screen SMC1A FS transcript in human tumor cell lines. 33 human breast tumor cell lines and non-tumorigenitic epithelial cell line MCF-10A and normal pancreatic epithelial cell line HPDE6. The experiment was performed by Dr. Buendia Jose Cano, CIM.

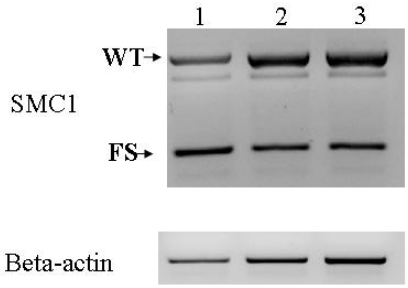


Figure AP2. 2 RT-PCR detection SMC1A FS transcript in normal BALB/C splenocytes. Splenocytes were separated by mouse $CD8^+$ T cell enrichment kit (Stemcell Technologies Inc, Vancouver, Canada). 1. $CD8^+$ splenocytes; 2. $CD8^-$ splenocytes; 3. B16F10 melanoma cell.

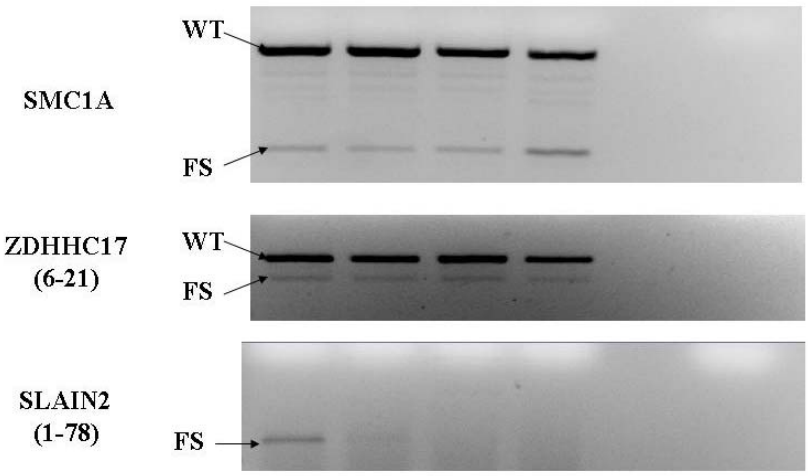


Figure AP2. 3 RT-PCR detection of SMC1A, 6-21 and 1-78 FS transcripts in four dog melanoma cDNAs.

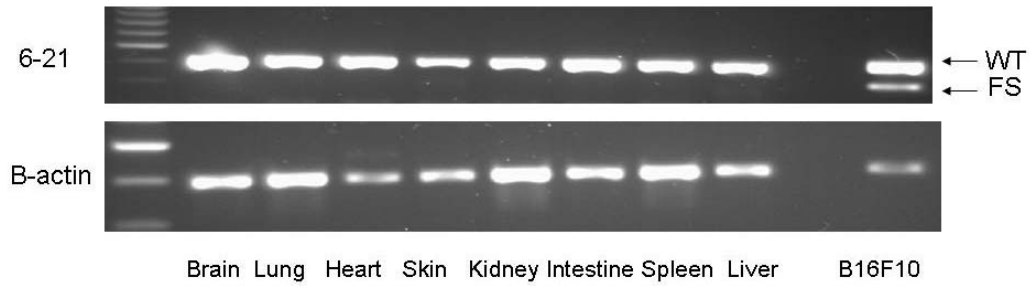


Figure AP2. 4 RT-PCR screen 6-21 FS transcript in C57BL6 mouse normal and B16F10 melanoma cell

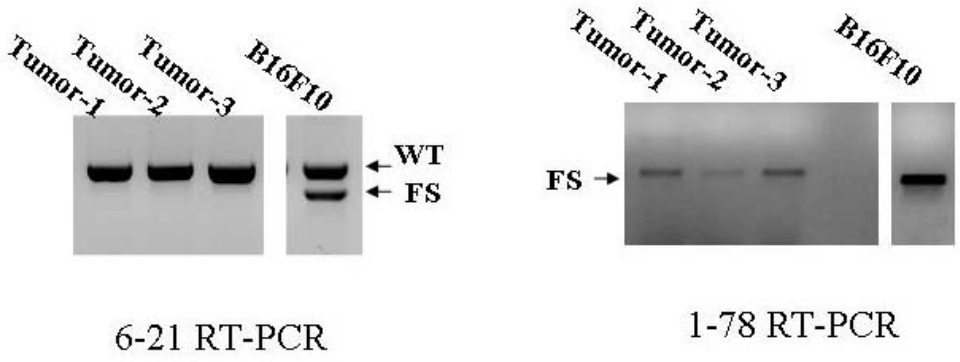
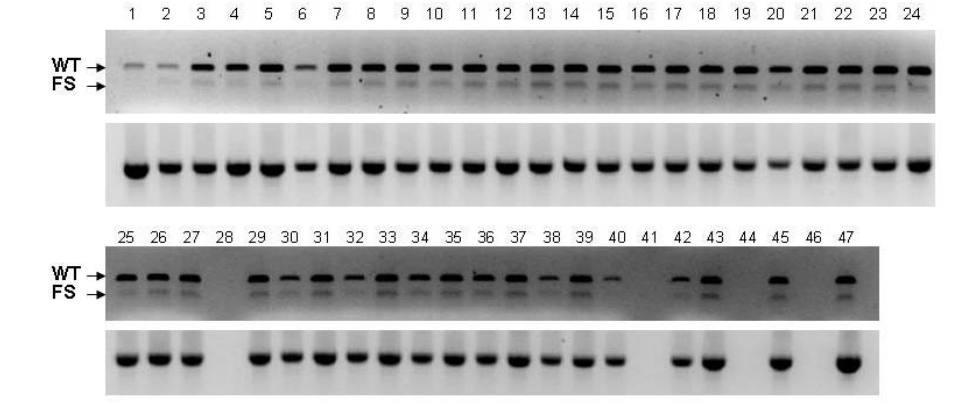


Figure AP2. 5 RT-PCR screen 6-21 and 1-78 FS transcript in spontaneous breast tumors from FVB/N-NeuT mice.



1	2	3	4	5	6	7	8	9	10	11	12
HTB132	CRL-1902	CRL 7940	CRL-2351	CRL 7368	CRL-2314	CRL 2322	CRL-2338	CRL 2335	CRL-1504	CRL 2324	CRL-2336
MDA-MB-468	UACC-893	SW 527	AU565	HS 606T	HCC38	HCC1187	HCC 1954	HCC1806	ZR-75-30	HCC1395	HCC1937

13	14	15	16	17	18	19	20	21	22	23	24
HTB 131	CRL-2327	CRL 2321	HTB-22	CRL 2914	HTB-126	CRL 2918	CRL2329	CRL-2326	CRL-7721	CRL-2315	CRL-7477
MDA-MB-453	HCC1428	HCC 1143	MCF-7	M4A4	Hs-578.T	NM2C5	HCC1500	HCC1419	MB157	HCC70	Hs739.T

25	26	27	28	29	30	31	32	33	34	35	36
HTB-123	CRL 10317	HTB-26		HTB-21	HTB 23	CRL-1500	HTB 128	CRL-7253	HTB 121	HTB-19	HTB 122
DU4475	MCF-10-A	MDA-MB-231		Cama-1	MDA-MB-134	ZR-75-1	MDA-MB-415	HS-362.T	BT483	BT-20	BT549

37	38	39	40	41	42	43	44	45	46	47	
CRL 2343	CRL 1897	CRL 2331	CRL 2316		CRL 7486	HTB 20		CRL-7245		CRL 7365	
HCC 2218	UACC 812	HCC 1599	HCC 202		Hs 748.T	BT474		Hs 343.T		Hs 605.T	

Figure AP2. 6 RT-PCR detection of 6-21 transcript in human breast tumors tumor cell lines and MCF-10A cell

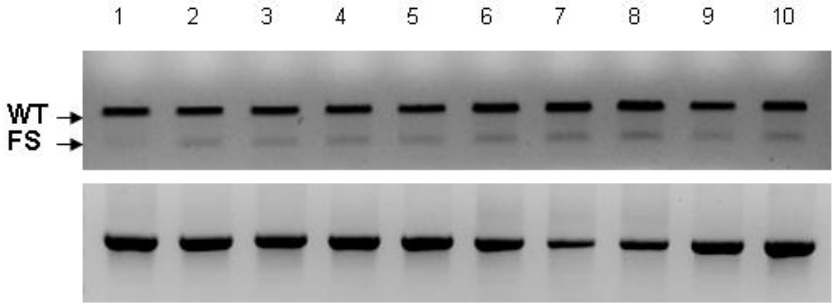


Figure AP2. 7 RT-PCR detection of 6-21 transcript in human primary breast tumors tumors and normal mammary tissues. 1-8: primary breast tumor cDNA, 9 and 10: normal mammary cDNA.

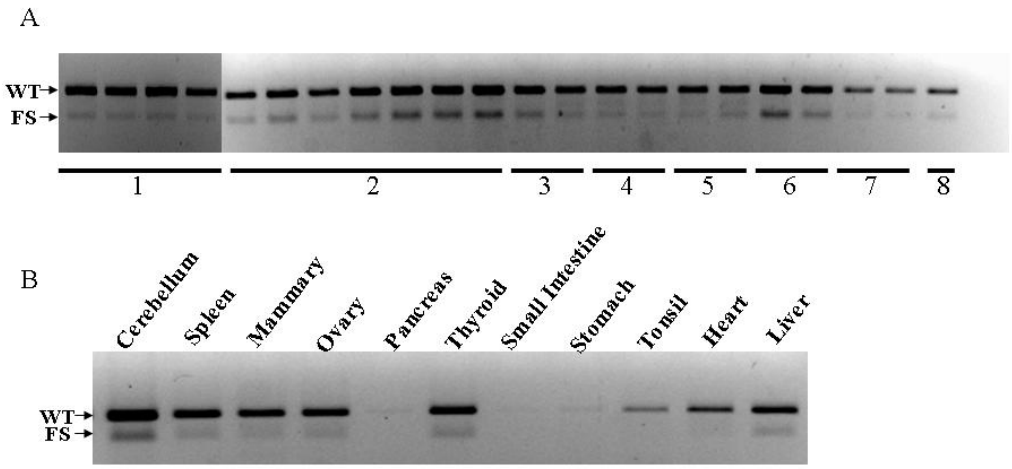


Figure AP2. 8 RT-PCR detection of 6-21 FS transcript in dog tumor cDNAs and normal tissues. A. Detection of 6-21 FS transcripts in dog tumor cDNAs. 1: Melanoma; 2: Osteosarcoma; 3. Lymphosarcoma; 4: Hemangiosarcoma; 5: Breast tumor; 6: Mast Cell Tumor; 7: Transitional cell carcinoma; 8: Thyroid adenocarcinoma. B. Detection of 6-21 FS transcripts in dog normal tissues.

APPENDIX 3

INSTITUTIONAL REVIEW BOARD (IRB)

ARIZONA STATE UNIVERSITY
Office of Research Integrity and Assurance
660 S. Mill Avenue Suite 315
Arizona State University
Tempe AZ 85287-6111
(Mail Code 6111)
Phone: 480-965-6788
Fax: (480) 965-7772

CONTINUING REVIEW FORM- IRB

- In accordance with Federal Regulations 45CFR46, the IRB must review nonexempt protocols at least annually, or more frequently if warranted.
- Please type your responses in the boxes provided. Use as much space as necessary (the boxes will expand). Please answer each question – if a question is not applicable, please put N/A in the box.
- Studies that are in the data analysis phase are considered open, researchers must complete this form.

1. Principal Investigator	
Principal Investigator: Stephen Albert Johnston	
ASU department address: Center for Innovations in Medicine, Biodesign Institute B230 MC5901	
E-mail address: Stephen.johnston@asu.edu	
Phone number: 480-727-0792	Fax Number: 480-727-0782
Co-Investigator(s) Name(s) and Contact Information: Phillip Stafford, Phillip.stafford@asu.edu; Kathryn Sykes, Kathryn.sykes@asu.edu; Lucas Restrepo, lucas.restrepo@asu.edu; Muskan Kukreja, muskan.kukreja@asu.edu	

2. Protocol Information
2a) Title of protocol: Profiling Human Sera for Unique Antibody signatures
2b) HS #: 0912004625
2c) If project is funded or funding is being sought, provide list of all sponsors and grant numbers: DTRA HDTRA1-11-1-0010; DoD BCRP W81XWHO710549; Please indicate the grant status for each source of funding: ☒ Active ☐ Pending
2d) ASU account number/project number: FQS0052, FQS0030
2e) Location(s) of research activity: Biodesign Center for Innovations in Medicine B225, B229, B233, B237
2f) IRB approval dates from additional institutions: All samples are provided to us from institutions that are current with their IRB approval. We currently do not have that information but can obtain if needed. **Please note that copies of current IRB approvals from additional institutions are required.*

3. Protocol Status
3a) Active: ☒ Yes ☐ No (If no, submit a close out report: http://researchintegrity.asu.edu/humans/forms
3b) Please indicate remaining duration of the study: 9 years
4. Participant Information
4a) Is this study closed to enrollment of new subjects: ☒ Yes ☐ No

4b) Total number of participants approved for the study (to be enrolled): N/A
4c) Number of participants enrolled (e.g. signed a consent form) during the **past approval period**: N/A
4d) Total number of participants enrolled **since study began**: N/A
4e) Total number of individuals screened (e.g. individuals that responded to study advertisements or other recruitment tractices and were questioned by investigators) in the past approval period (if applicable): N/A (this includes the number that was later enrolled)
4f) Of the total number of individuals screened in the past approval period, what percentage has been ineligible to participate in the study (if applicable)? N/A
4g) Number of enrolled participants who withdrew from the study: N/A Please state the reason(s) the participant(s) withdrew.
4h) Number of participants still to be enrolled: N/A (If this brings the sample to greater than what is listed in 4b, submit a request for modification see 7d).
4i) Participant enrollment breakdown by gender, age and ethnicity: (This information is required for all studies that are NIH-sponsored. It is recommended, but not required, that other researchers provide this information). N/A

5. Data Sources Check all categories that apply to your protocol:	
☐	Human subjects intervention with use of informed consent form
☐	Discarded, identified pathological materials, no intervention
☐	Genetic analysis
☐	Interviews or questionnaires
☐	Medical records or other records from human subjects
☒	Other please specify: **We have clinical diagnosis and treatment status, genotype information and maybe smoking history. All information though is provided to us.**

6. Adverse Events or Unexpected Problems

6a) Have there been any complaints from subjects in the past approval period?
☐ Yes If yes, describe ☒ No

6b) Have there been any ***adverse events*** or unexpected problems in the past approval period?
☐ Yes ☒ No
If yes, please explain in detail and indicate when the IRB was notified of the event or problem. If the IRB was not notified, please explain why this was not done.

6c) Does the study have a Data Safety Monitoring Board (DSMB)? ☐ Yes ☒ No
If yes, please indicate the date of the last DSMB review:

Please note that investigators are required to submit DSMB reports to the ASU IRB at the time they are made available to the investigator.

7. Protocol Modifications or Revisions

7a) Have there been any modifications or revisions to the protocol in the past approval period?
☐ Yes ☒ No
If yes, please indicate the date of the approval from the Committee for the modification or revision and provide a brief description.

7b) Have there been any deviations from the approved protocol? ☐ Yes ☒ No
If yes, please describe to self-report the protocol violation.
7c) Do you want to add any new co-investigators to the study? ☐ Yes ☒ No
If yes, submit their names and copies of the human subjects training required by the IRB:
http://researchintegrity.asu.edu/training/humans
7d) Do you wish to submit a modification at this time? ☒ Yes ☐ No
If yes, please describe the modification request and rationale for the changes: Please remove Dr. Patricia Carrigan from the protocol. She has left ASU.

8. Current Consent Form

8a) Please attach a copy of your current consent form for renewal if you are enrolling new subjects. N/A

8b) Is this the original consent form or a revised form? ☐ Original ☐ Revised (If revised, please provide date of ASU IRB approval for the revision. Attach a copy of the stamped form and unstamped form)

9. Protocol Progress Report

9) Please submit a ***detailed*** progress report. The progress report must be substantive and complete, and include the goal(s) of the study, findings to-date, how data is being stored, and plans for the next year/review period. If this project is funded, please send a copy of the most recent progress report that was sent to the funding agency:

The last year, our team has optimized the immunosignature microarray and has contracted with Applied Microarrays (AMI) in Tempe, AZ to print our arrays. We obtained a new set of 10,000 different random peptides, as the last set had been depleted. We ensured that the new peptides were carefully diluted in a new buffer/organic mix that is compatible with AMI's printing process. The added precision of commercial printing has allowed us to obtain higher reproducibility across patients, and find much more subtle changes in antibody responses. We have completed the Valley Fever project by printing a set of 100-peptide 'diagnostic arrays' to do the test-training sample sets. We have obtained 65 look-back blinded samples from John Galgiani at U of A in Tucson that were all false negative samples from his clinic. We classified these samples with 0% error (after excluding problematic samples that were inherently high-background or had been subject to degradation effects). We are in the process of writing these data up in a manuscript.

We completed a project on glioblastoma multiformae, using blinded samples from Barrow Neurological Institute (BNI) in which we were able to identify brain cancer grade as well as presence or absence of an important methylation enzyme, MGMT. This enzyme's status has been shown to be an effective predictor of response to Temozolamide. We have submitted this manuscript to NeuroOncology.

We have completed a project on Esophageal Cancer, using blinded samples obtained from Mayo Clinic, in which we were able to distinguish presence or absence of Esophageal cancer in patients. We are currently examining samples from patients with Barrett's Esophagus, to determine whether we can detect early cancer predisposition.

We have built a pathogen microarray, in which 5K peptides from human pathogens were tiled on a standard glass slide. We are currently optimizing this platform to distinguish patients who are convalescent from one or another infectious agent. We have found that printing methods that enhance the immunosignaturing effect are deleterious to the discrimination of our pathogen epitope arrays. Thus we are altering the printing characteristics for these arrays, and are using a slide surface that spaces the peptides out much further than our aminosilane slides allow.

10. Publications, Presentations and Recent Findings

10a) Have there been any presentations or publications resulting from this study during the past approval

period? ☒ Yes ☐ No If yes, please submit a copy of the abstract, or the publication, with this application.

"Immunosignaturing can detect products from molecular markers in brain cancer"– submitted to NeuroOncology
"Physical parameters Affecting Antibody Profiles as Biomarkers of Health Status" – revision resubmitted to Molecular and Cellular Proteomics
"Sample Preparation for Immunosignaturing" – revision resubmitted to Vaccine

Presentations:
BRP FY11 Vision Setting Meeting, November 2010 – Panel Member
3rd Annual Oncology Biomarker Conference, January 2011 – Invited speaker
Leading Innovation and Knowledge Sharing (LINKS) BCMRP meeting, February, 2011 - Panel member
BCRP FY10 Programmatic Review Meeting, March 2011 – Panel member
Canary Foundation, March 2011 – Invited participant
Era of Hope Abstract Placement Meeting, April 2011 – Committee member
NBCC Artemis Project, April 2011 – Workshop participant
Era of Hope Meeting, Orlando, August 2011 – Invited speaker, Organizing committee member

10b) Have there been any recent findings either from this study, or a related study (through a literature review for example), that would have an effect on this study's risk/benefit analysis? ☐ Yes ☒ No
If yes, please describe and cite references:

11.Conflicts of Interest and Commercialization

11. Does any member of the research team have a potential conflict of interest with this study that could affect study participants and/or study outcome? For more information about examples of conflicts of interests, please visit the ASU objectivity website: http://researchintegrity.asu.edu/coi
☐ Yes (If yes, please describe and disclose in the consent form) ☒ No

b) Does the PI or Co-I have a current conflict disclosure form on file at the ASU Office of Research Integrity and Assurance?
☐ Yes ☐ No

c) If there are conflicts of interests, please describe the ways in which you have and will minimize harm to research subjects and/or the objectivity of research. No prospective human trials have been proposed, only blinded retrospective samples are currently being run on the immunosignaturing platform.

12. Training

12.The research team must verify completion of human subjects training within the last 3 years. (http://researchintegrity.asu.edu/training/humans)

CITI training – Provide the date that the PI and Co-I's completed the training:
If you completed **NIH** training prior to 9/15/10 this will be accepted. Provide a copy of the certificate.

13. Required Signatures

Revised 8/11

Principal Investigator:  Date: 10/14/11

FOR IRB USE
Chair or Committee member name:

Signature: Date:

Revised 8/11

ASU Knowledge Enterprise Development

Office of Research Integrity and Assurance

To:	Stephen Johnston BDB
From:	Carol Johnston, Chair Biosci IRB
Date:	10/24/2011
Committee Action:	**Renewal**
Renewal Date:	10/24/2011
Review Type:	Expedited F5
IRB Protocol #:	0912004625
Study Title:	Profiling Human Sera for Unique Antibody Signatures
Expiration Date:	11/21/2012

The above-referenced protocol was given renewed approval following Expedited Review by the Institutional Review Board.

It is the Principal Investigator's responsibility to obtain review and continued approval of ongoing research before the expiration noted above. Please allow sufficient time for reapproval. Research activity of any sort may not continue beyond the expiration date without committee approval. Failure to receive approval for continuation before the expiration date will result in the automatic suspension of the approval of this protocol on the expiration date. Information collected following suspension is unapproved research and cannot be reported or published as research data. If you do not wish continued approval, please notify the Committee of the study termination.

This approval by the Biosci IRB does not replace or supersede any departmental or oversight committee review that may be required by institutional policy.

Adverse Reactions: If any untoward incidents or severe reactions should develop as a result of this study, you are required to notify the Biosci IRB immediately. If necessary a member of the IRB will be assigned to look into the matter. If the problem is serious, approval may be withdrawn pending IRB review.

Amendments: If you wish to change any aspect of this study, such as the procedures, the consent forms, or the investigators, please communicate your requested changes to the Biosci IRB. The new procedure is not to be initiated until the IRB approval has been given.

ASU Knowledge Enterprise Development

Office of Research Integrity and Assurance

Biosci IRB

To: Johnston,Stephen Albert **Date:** 09 / 26 / 2011

From: Biosci IRB **Expiration Date:** 11 / 22 / 2011

Re: **Protocol #** 0912004625: Profiling Human Sera for Unique Antibody Signatures

This letter serves as a IRB notification reminder by the Biosci IRB. It is the primary responsibility of the Principal Investigator to ensure that the re-approval status for lapsed protocols is achieved. All protocols must be re-approved annually by the IRB unless shorter intervals have been specified.

Please note that the level of review given to the continuing review process is the same as that of any new protocol. All requests for re-approval must be reviewed at a convened IRB meeting, except for those protocols that meet the criteria for expedited review.

Please submit the following documents at least three weeks prior to the expiration date to allow for full committee review:

1) A completed Continuing Review Form.

2) Two (2) copies of each consent form(s) used in the study (If data collection is ongoing).

Please note that you can obtain a copy of the Continuing Review Form through our web site: http://researchintegrity.asu.edu/humans.

As of July 1, 2003, all personnel involved in human subjects research must complete the Human Subjects training course. It is the responsibility of the Principal Investigator to make sure all personnel associated with this study have completed the human subjects training course (see the Office of Research Integrity and Assurance website for a link to the NIH training).

It is a violation of Arizona State University policy and federal regulations to continue research activities after the approval period has expired. If the IRB has not reviewed and re-approved this research by its current expiration date, all enrollment, research activities and intervention on previously enrolled subjects must stop. If you believe that the health and welfare of the subjects will be jeopardized if the study treatment is discontinued, you may submit a written request to the IRB to continue treatment activities with currently enrolled subjects.

Your assistance and cooperation in ensuring that the above-mentioned protocol is received for re-approval evaluation at the Office of Research Integrity and Assurance before the lapse date is greatly appreciated.

Sent to Office of Research Integrity 10/18/11

APPENDIX 4

INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

utional Animal Care and Use Committee (IACUC)
izona State University

Tempe, Arizona 85287-3503
(480) 965-2179 FAX: (480) 965-8013

Animal Protocol Review

Protocol Number: 05-817R
Protocol Title: Genetic Cancer Vaccines
Principal Investigator: Stephen Johnston
Date of Action: 06/17/2005 Final Action Date: 06/17/2005

The animal protocol review was considered by the Committee and the following decisions were made:

- ☐ The original protocol was APPROVED as presented.
- ☒ The revised protocol was APPROVED as presented.
- ☐ The protocol was APPROVED with RESTRICTIONS or CHANGES as listed below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
- ☐ The Committee requests CLARIFICATIONS or CHANGES in the protocol as described below. Approval is contingent upon review and approval of the required revisions by the IACUC Chair.
- ☐ The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
- ☐ The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
- ☐ The Committee requests you to contact _____ to discuss this proposal.
- ☐ A copy of this correspondence has been sent to the Vice President for Research.

RESTRICTIONS, CHANGES OR WAIVER REQUIREMENT:

Approved Number of Animals: 3,000 Mice

Approval Period: 06/17/2005 – 06/16/2008

Signature: _____ Date: 06/17/2005
IACUC Chair or Designee

Investigator cc: IACUC Office, IACUC Chair, ORSPA

VI. DUPLICATION AND ALTERNATIVES

A. Provide the following details for the most recent literature search used to explore for duplicative research, alternatives to painful procedures and most currently relevant teaching use of animals.

Date that search was conducted: 4/5/2005
Database used: Medline
Publication years covered by the search: Last 5 years
Keywords used: Genetic immunization, cancer vaccine, tumor antigens, melanoma, and breast cancer

B. Describe any other procedures (e.g., participation in meetings, review of journals) that are used to evaluate duplication and explore alternatives:

We will continue to monitor commercial sources should one be available. In addition, we will monitor scientific literature for comparable reagents as needed. Journals that we routinely monitor include Science, Nature groups, Vaccine, PNAS, EMBO, Cancer Research, to name a few. In addition, Dr. Johnston attends multiple scientific meetings each year to keep abreast of new developments.

C. Does this research replicate previous work? **NOTE: Teaching protocols need not address item VI.c.**

☑ No. Proceed to section VII.
☐ Yes. Explain why the replication is necessary:

VII. ASSURANCE:

The information contained herein is accurate to the best of my knowledge. I have carefully compared the proposed work with the current state of knowledge in this field by reviewing the literature and it is my professional opinion that the proposed work meets high standards of scientific merit. If the study involves pain and distress to the animal, whether or not it is relieved by anesthetics or analgesics, I have (1) reviewed the literature related to this work and have found no significant studies which could make this protocol unnecessarily duplicative, and (2) considered alternatives to animal use and found none available, as described above. Procedures involving animals will be carried out humanely and all procedures will be performed by or under the direction of trained or experienced persons. Any revisions to animal care and use in this project will be promptly forwarded to the Animal Care and Use Committee for review. Revised protocols will not be used until Committee clearance is received. The use of alternatives to animal models has been considered and found to be unacceptable at this time.

The principal investigator, by signing below, and the IACUC recognize that other medications may be given to the animals for veterinary care purposes (including humane euthanasia of animals in pain that cannot be controlled, as determined by the University Veterinarian or a euthanasia-certified principal investigator).

Individual listed on I.A. — Date: 6/30/05

Stephen Albert Johnston, Director CIM — Date: 6/30/05
Insert Name and Title Here – copy and paste as necessary

***Department Chair — Date

***College Dean — Date

***ASU East requires these signatures.

NOTE: Principal investigators are requested to attach a two-page biosketch reflecting their most recent pertinent experience.

Institutional Animal Care and Use Committee (IACUC)
Arizona State University

Tempe, Arizona 85287-1103
(480) 965-2179 FAX: (480) 965-7772

Animal Protocol Review

ASU Protocol Number: 08-1000R
Protocol Title: Genetic Cancer Vaccines
Principal Investigator: Stephen Johnston
Date of Action: 07/01/2008

The animal protocol review was considered by the Committee and the following decisions were made:

- ☒ The original protocol was APPROVED as presented.
- ☐ The revised protocol was APPROVED as presented.
- ☐ The protocol was APPROVED with RESTRICTIONS or CHANGES as noted below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
- ☐ The Committee requests CLARIFICATIONS or CHANGES in the protocol as described in the attached memorandum. The protocol will be reconsidered when these issues are clarified and the revised protocol is submitted.
- ☐ The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
- ☐ The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
- ☐ The Committee requests you to contact _____ to discuss this proposal.
- ☐ A copy of this correspondence has been sent to the Vice President for Research.
- ☐ Amendment was approved as presented.

Approved # of Animals: 6,336 Mice **Pain Level:** D
Approval Period: 07/01/2008 - 06/29/2011
Funded: Department of Defense
Title: Towards Developing a Prophylactic Breast Cancer Vaccine

Signature: _____ Date: 7/1/08
IACUC Chair or Designee

Original: Principal Investigator
cc: IACUC Office
IACUC Chair
ORSPA/SPS

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 315
Tempe, Arizona 85287-6111
Phone: (480) 965-4387 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number: **11-1197R**
Protocol Title: **Genetic Cancer Vaccines**
Principal Investigator: **Stephen Johnston**
Date of Action: **06/24/2011**

The animal protocol review was considered by the Committee and the following decisions were made:

- ☐ The original protocol was APPROVED as presented.
- ☒ The revised protocol was APPROVED as presented.
- ☐ The protocol was APPROVED with RESTRICTIONS or CHANGES as noted below. The project can only be pursued, subject to your acceptance of these restriction or changes. If you are not agreeable, contact the IACUC Chairperson immediately.
- ☐ The Committee requests CLARIFICATIONS or CHANGES in the protocol as described in the attached memorandum. The protocol will be considered when these issues are clarified and the revised protocol is submitted.
- ☐ The protocol was approved, subject to the approval of a WAIVER of provisions of NIH policy as noted below. Waivers require written approval from the granting agencies.
- ☐ The protocol was DISAPPROVED for reasons outlined in the attached memorandum.
- ☐ The Committee requests you to contact _____ to discuss this proposal.
- ☐ A copy of this correspondence has been sent to the Vice President for Research.
- ☐ Amendment was approved as presented.

RESTRICTIONS, CHANGES OR WAIVER REQUIREMENTS:

Total # of Animals: **9,024** **Pain Level:** **B-720; C-3,074; D-5,230** **Species:** **Mice**
Sponsor: **Department of Defense**
Title: **Towards Developing a Prophylactic Breast Cancer Vaccine**
Proposal #: **W81XWH0710549**
Approval Period: **06/24/2011 – 06/23/2014**

Signature: _____ Date: 6/24/11
IACUC Chair or Designee

Original: Principal Investigator
Cc: IACUC Office
IACUC Chair

Date: 6/7/2012

ARIZONA STATE UNIVERSITY
IACUC ANNUAL REVIEW

I. Currently approved protocol

Protocol Number: 11-1197R
Protocol Title: Genetic Cancer Vaccine
Principal Investigator: Stephen Johnston

II. Status of Project

A. Was the research or teaching conducted?

i. ☐ No. If no,
1. Will the protocol be terminated?

a. ☐ Yes. Proceed to item VI.
b. ☐ No. Proceed to item II B.

ii. ☒ Yes. If yes,
1. Were there any significant animal welfare issues (morbidity or mortality, complications, etc.) encountered over the past 12 months?

a. ☒ Yes. Please describe (include the problem, approximate number of animals affected, and resolution). Proceed to item II B when completed.
About 5% of the FVB/N-NeuT females spontaneously died. This is a common characteristic of this transgenic mouse strain. All of the other mice behaved normally.

b. ☐ No. Proceed to item II B.

B. Will the research or teaching continue with no anticipated protocol changes in animal species, animal numbers, or categories listed below for the next 12-month period?

- Procedures
- Criteria to Measure/Monitor Pain or Distress
- Alternatives to Painful Procedures
- Restraint
- Amelioration and Control of Painful Procedures
- Estimation of Potential Postoperative/Intervention Pain
- Postoperative/Chronic Care
- Euthanasia/Disposition of Animals
- Animal Care and/or Use Sites

i. ☒ Yes. Proceed to item III.
ii. ☐ No. If there will be proposed changes, you must complete an Amendment Request form describing all proposed changes as well as the scientific rational for these changes. Proceed to item III.

III. Updated Information

A. Please evaluate the Category of Pain as stated in your currently approved protocol. Do you feel it remains appropriate for the procedures performed?

i. ☒ Yes. Proceed to item III B.
ii. ☐ No. If no, please describe: Proceed to item III B when completed.

B. Have there been any recent findings, either from this study or a related study, that would change the planned use of animals?

i. ☐ Yes. If yes, cite references below or in an attachment and submit an Amendment Request form. Proceed to item IV when completed.

ii. ☒ No. Proceed to item IV.

IV. Progress Report

Provide a statement on progress of your teaching or research under this protocol over the past 12 months. Include any presentations or publications that have resulted from this protocol during the past 12 months.

We optimized the immunization regime for our prophylactic cancer vaccine candidates. The protection by individual and pool FS antigens were confirmed in both FVB/N-NeuT and BALB-NeuT mice models. We are currently working on further optimization of the immunization to achieve the additive protection by pooling more FS antigen candidates. We presented "Frameshift Peptides as Prophylactic Cancer Vaccines Antigens" at 2012 annual meeting of American Association for Cancer Research at April 2012.

V. Personnel

All personnel who work with animals are required to have animal care training within the last three years. ASU IACUC training modules can be completed at the LATA ASU homepage. Training dates can also be verified by users at this site: **http://balsam.forest.net/latanet/records/asut/search3.htm**

A. List the names, titles, affiliations, and roles of **ALL** persons currently involved in the research or teaching activity.

Name	Title	ASURITE name	Role in Protocol (What procedures will each person be doing?)	Species with which individual will have direct contact ("all" or list species)*	IACUC USE ONLY Training (mm/yy)
Stephen Johnston, Ph.D.	PI, Center Director, CIM		Design experiments	None	4/10 HSQ
Kathryn Sykes, Ph.D.	Adjunct Professor		Design Experiments, interpret data, troubleshoot	None	11/11 HSQ
Christopher Diehnelt, Ph.D.	Res. Asst. Professor		Design Experiments	None	Basic 9/09, Mice 4/10 HSQ
Danielle Lussier	Graduate Student		Immunization, /bleed mice /Euthanasia	Mouse	HSQ 11/11
Andrey Loskutov, Ph.D.	Research Scientist		Immunization, /bleed mice /Euthanasia	Mouse	2/11 HSQ

Luhui Shen	Graduate Student		Immunization, /bleed mice /Euthanasia/Breed Tg mice/tumor cell injection/monitoring	Mouse	11/11 HSQ
Felicia Craciunescu	Researcher		Immunization, /bleed mice /Euthanasia/tumor cell injection/monitoring	Mouse	10/09 HSQ
John Charles Rodenberry	Researcher		Immunization, /bleed mice /Euthanasia/Breed Tg mice/tumor cell injection/monitoring	Mouse	2/11 HSQ
Kurt Whittemore	Graduate Student		Immunization, /bleed mice /Euthanasia/Breed Tg mice/tumor cell injection/monitoring	Mouse	6/12 HSQ
Kari Kotlarczyk	Technician		Immunization, /bleed mice /Euthanasia/Breed Tg mice/tumor cell injection/monitoring	Mouse	7/10 HSQ
Hu Duan	Graduate Student		Immunization, /bleed mice /Euthanasia/Breed Tg mice/tumor cell injection/monitoring	Mouse	6/10 HSQ

B. List the names of any individuals no longer involved with the research (these individuals will be removed from the protocol and DACT will be notified):
Mark Robida, Kristen (Day) Seifert

VI. Certification

By signing this report, I certify that, to the best of my knowledge, the information included herein is accurate and complete. I understand that continued animal use past the scheduled termination date of the protocol requires IACUC approval. I also understand that should the animal use under this protocol require any change from that stated in the protocol, prior approval by the IACUC is required.

Principal Investigator's Signature

6/7/12
Date

FOR IACUC USE ONLY
Annual Review Determination

ANNUAL REVIEW APPROVAL SIGNATURES:

Chair, IACUC — Date: 6-28-12

Attending Veterinarian — Date: 6-28-12

Additional IACUC Member — Date: 6-28-12